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(54) **ANTI-CD40 MONOCLONAL ANTIBODY**

(57) An antibody or a functional fragment thereof, acting agonistically or antagonistically on CD40.

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## Description

## TECHNICAL FIELD

**[0001]** The present invention relates to an antibody or a functional fragment thereof that recognizes a human CD40 antigen present on the surface of human B cells, dendritic cells (DC) and the like. Specifically, the present invention relates to an anti-human CD40 antibody or a functional fragment thereof that is substantially antagonistic to a human CD40 antigen on the dendritic cell (DC) surface, and an agonistic anti-human CD40 antibody or a functional fragment thereof that is expected to have a therapeutic effect higher than those of conventional anti-human CD40 antibodies.

## BACKGROUND ART

## 1. CD40

**[0002]** CD40 is an antigen with a molecular weight of 50 kDa that is present on the cell membrane surface. CD40 is expressed on B cells, dendritic cells (DC), certain types of cancer cells, and thymic epithelial cells. CD40 is known to play a key role in proliferation and differentiation of B cells and DC. CD40 has been identified as an antigen that is expressed on the human B cell surface (E. A. Clark et. al., Proc. Natl. Acad. Sci. USA 83: 4494, 1986, 1. Stamenkovic et. al., EMBO J. 8:1403, 1989). Based on the amino acid sequence homology, CD40 is thought to be a member of the TNF receptor family, to which a low affinity NGF receptor, TNF receptor, CD27, OX40, CD30 and the like belong. The gene of a ligand (CD40L) for human and mouse CD40 has been cloned recently, revealing that it is a type II membrane protein, and is expressed on activated CD4<sup>+</sup>T cells. It has also been shown that CD40L introduces strong activation signals into human and mouse B cells.

**[0003]** The expression of CD40 has been confirmed more often on dendritic cells than on B cells, so that it has become clear that CD40 plays an important role. The binding of CD40 with CD40L causes the activation of antigen-presenting cells (APC). Specifically, it enhances the expression of co-stimulation molecules such as CD80 (B7-1) and CD86 (B7-2), or the production of IL-12 (Caux, C., et al.: Activation of human dendritic cells through CD40 cross-linking. J. Exp. Med., 180:1263, 1994), (Shu, U., et al: Activated T cells induce interleukin-12 production by monocyte via CD40-CD40 ligand interaction. Eur. J. Immunol. 25: 1125, 1995). Dendritic cells show strong antigen-presenting ability, and have strong helper T (Th) cell-activating ability. Furthermore, it is thought that dendritic cells control the differentiation of naive Th cells into Th1 or Th2 cells. When dendritic cells (DC1) are made to mature by culturing peripheral blood monocytes that are myeloid dendritic cells in the presence of GM-CSF and IL-4 and using CD40L, the DC1 in vitro are capable of producing IL-12, stimulate and activate allo-naive Th cells, and thus induce IFN $\gamma$ -producing T cells (specifically, promotes differentiation into Th1). Since this action is inhibited by anti-IL-12 antibodies, the reaction may be mediated by IL-12. On the other hand, when lymphocyte-dendritic cells (DC2) are prepared by culturing lymphatic tissue T regions or plasmacytoid T cells present in peripheral blood with IL-3 and CD40 ligands, DC2 are incapable of producing IL-12, stimulate and activate allo-naive Th cells, induce IL-4-producing T cells, and thus promote differentiation into Th2. It is thought that Th1 cells are involved in the activation of cellular immunity, and Th2 cells are involved in enhancement of the ability for humoral immunity as well as the suppression of the ability for cellular immunity. Cytotoxic T cells (CTL) activated with the help of Th1 cells can remove causative factors (many viruses, *Listeria monocytogenes*, *tubercle bacillus*, toxoplasma protozoa and the like) multiplying in the cytoplasm and tumor cells.

**[0004]** It has been shown that anti-CD40 monoclonal antibodies that recognize CD40 expressed on the membrane surfaces exert a variety of biological activities on B cells. Anti-CD40 monoclonal antibodies are largely classified into agonistic and antagonistic antibodies impacting the interaction between CD40 and CD40L.

## 2. Agonistic antibody

**[0005]** The activation of B cells is known as an action of agonistic antibodies. For example, anti-CD40 antibodies have been reported to induce cell adhesion (Barrett et al., J. Immunol. 146: 1722, 1991; Gordon et al., J. Immunol. 140: 1425, 1988), enhance cell size (Gordon et al., J. Immunol. 140: 1425, 1988; Valle et al., Eur. J. Immunol. 19: 1463, 1989), induce the division of B cells that are activated only with anti-IgM antibodies, anti-CD20 antibodies or phorbol ester (Clark and Ledbetter, Proc. Natl. Acad. Sci. USA 83: 4494, 1986; Gordon et al., LEUCOCYTE TYPING III. A. J. McMichael ed. Oxford University Press. Oxford, p. 426; Paulie et al., J. Immunol. 142: 590, 1989), induce the division of B cells in the presence of IL4 (Valle et al., Eur. J. Immunol. 19: 1463, 1989; Gordon et al., Eur. J. Immunol. 17: 1535, 1987), induce the expression of IgE (Jabara et al., J. Exp. Med. 172: 1861, 1990; Gascan et al., J Immunol. 147: 8, 1991), IgG and IgM (Gascan et al., J. Immunol. 147: 8, 1991) of cells stimulated with IL-4 and cultured without T cells, enhance the secretion and the on-the-cell expression (Challa A, Allergy, 54: 576, 1999) of soluble CD23/Fc $\epsilon$ RII from B cells by IL-4 (Gordon and Guy, Immunol. Today 8: 339, 1987; Cairns et al., Eur. J. Immunol. 18: 349, 1988),

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and promote IL-6 production (Clark and Shu, J. Immunol. 145: 1400, 1990). Furthermore, it has been reported that B cell clones are established from human primary culture B cells by adding IL-4 and anti-CD40 antibodies in the presence of CDw32+ adhesion cells (Bancherou et al., Science 241:70,1991), and the inhibition of the apoptosis of germinal center cells is mediated by CD40, regardless of the function of antigen receptors (Liu et al., Nature 342: 929,1989). As described above, CD40 has been identified as an antigen expressed on the human B cell surface. Thus, most of the isolated antibodies have been evaluated mainly using function to induce the proliferation and differentiation of human B cells and activity to induce cell death in cancer cells as indicators (Katira, A. et. al., LEUKOCYTE TYPING V. S. F. Schlossman, et al. eds. p. 547. Oxford University Press. Oxford, W. C. Flansow et al., LEUKOCYTE TYPING V. S. F. Schlossman, et al. eds. p. 555. Oxford University Press. Oxford, J. D. Pound et al., International Immunology, 11: 11, 1999).

**[0006]** Anti-CD40 antibodies were shown to cause the maturation of DC (Z. H. Zhou et. al., Hybridoma, 18: 471 1999). Moreover, the role of CD4T cells in antigen-specific CD8T cell priming has been reported to activate DC via CD40-CD40L signaling. It was shown that the role of CD4 helper T cells in activation of dendritic cells (DC) can be replaced by that of anti-CD40 monoclonal antibodies (mAb) (Shoenberger, S.P., et al.: T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature, 480, 1998). Furthermore, it was shown in mice that the organism can be protected not only from tumor cells expressing CD40 but also from tumor cells not expressing the same by the administration of anti-CD40 antibodies (French, R. R., et. al.: CD40 antibody evokes a cytotoxic T-cell response that eradicates lymphoma and bypasses T-cell help. Nature Medicine, 5, 1999).

**[0007]** Most antibodies reported to date have not been isolated using the effect on DC as an indicator. However, in terms of the modification of DC functions, antibodies selected by their action on B cells are likely to be insufficient as therapeutic agents. It was reported that among monoclonal antibodies against mouse CD40, there are clones that react to DC, but do not react to vascular endothelial cells, and, conversely, clones that do not react to DC, but react to vascular endothelial cells, depending on epitopes that the antibodies recognize (Van Den Berg, TK, et. al., Immunology, 88: 294, 1996). It is also assumed that the binding and action of human CD40 antibodies to DC differ depending on epitopes.

**[0008]** It is known that anti-CD40 antibodies or CD40 ligands can suppress the proliferation of CD40-expressing lymphoma cell lines and thus can induce the cell death (Funakoshi S et al., Blood, 83: 2782, 1994; Funakoshi S et al., Journal of Immunotherapy, 19, 93, 1996; Z. H. Zhou et. al., Hybridoma, 18: 471 1999; and Joseph A et al., Cancer Research, 60: 3225, 2000). What is interesting about agonistic antibodies is that the function of the antibody does not always coincide always with that of CD40L. Action to activate B cells does not also coincide with action to suppress B cell tumor growth. It is desired to develop antibodies having both DC-activating ability and tumor cell proliferation-suppressing action. Moreover, among agonistic antibodies, both antibodies that inhibit and those that do not inhibit the binding of CD40L to CD40 are present (Challa A et al., Allergy, 54: 576, 1999). For example, antibodies produced by G28-5 (ATCC No.HB-9110) compete with CD40L, so that there is no effect resulting from the combined use with CD40L. The degree of activation of CD40-expressing cells differs depending on antibodies. Even when antibodies exhibit independently weak agonistic activity, the combined use of the antibodies with CD40 ligands may more significantly promote the activity in the presence of the antibodies, than the activity resulting from CD40 ligands alone. In contrast, even when antibodies exhibit independently agonistic activity, inhibition of CD40 ligands may lower the activity in the presence of the antibodies to a greater extent than the activity resulting from CD40 ligands alone (Pound et al., International Immunology, 11:11, 1999). It was shown that with antibodies that do not compete with CD40 ligands, stronger suppression of proliferation can be achieved in the presence of CD40 ligands, although the tumor cell proliferation-suppressing action of the antibody itself is weak (Joseph A et al., Cancer Research, 60: 3225, 2000). Accordingly, it is desired to develop antibodies that bind to CD40 to suppress independently cell proliferation, but that do not inhibit the binding of CD40 ligands to CD40. By taking full advantage of such characteristics, there is a possibility of developing a therapeutic agent that is more efficient than a soluble CD40L. For example, the soluble CD40L is activated by binding with CD40, and at the same time, it suppresses the function of CD40L present in vivo. An antibody that does not compete with CD40L, does not cause such suppression, and has better therapeutic effects can be expected by synergistic effect.

### 3. Antagonistic antibody

**[0009]** In the meantime, as described above, it is expected that because CD40 plays an important role in immune reaction, therapeutic agents for immune suppression upon organ transplantation and against autoimmune disease can be developed by inhibiting the binding of CD40 with its ligand. Sawada-Hase et al., have reported that the proportion of cells strongly expressing CD40 was increased in the peripheral blood monocytes of Crohn's disease patients. However, antibodies that inhibit the binding of CD40 with its ligand have not been well understood. For example, such antibodies that inhibit the binding may be effective for the functional analysis of CD40, and therapy against disease, for which activation of CD40 is required. Moreover, antibodies that inhibit CD40 ligands have been also shown to have

the potential of being effective as agents against diseases with which the binding of CD40 with CD40 ligands is involved. However, it has been reported that CD40L is expressed in activated blood platelets (V. Henn et. al., Nature 391: 591, 1998). Thus, it has been reported that there is a risk of causing thrombi, if anti-CD40L antibodies are used as a therapeutic agent (T. Kawai et. al., Nat. Med. 6: 114, 2000). From such a point of view, antibodies against CD40 can be expected to be safer than anti-CD40L antibodies, as an antibody therapeutic agent that inhibits the binding of CD40 with its ligand. Anti-CD40 antibodies are required to suppress the binding of CD40L to CD40, and not to activate CD40 by the antibody itself.

[0010] Although a huge number of studies have been conducted in the past concerning antibodies that bind specifically to human CD40 and suppress the binding of CD40L to CD40 without activating CD40, only a single case, that is a mouse anti-human CD40 antibody, named 5D12, has been reported (J. Kwekkeboom et al., Immunology 79: 439, 1993). In addition, it has not been known whether or not antibodies showing neutralization activity for B cells can also show the same for DC that is, if the antibodies can neutralize the action of CD40 ligands. Furthermore, it has been reported that the action of biotinylated anti-mouse CD40 antibodies is enhanced by cross-linking with avidin (Johnson et al., Eur J Immunol, 24: 1835, 1994). We enhanced the action of soluble CD40 ligands against a B cell line (Ramos cells) using antibodies (M2) against tags (FLAG), which had been previously provided by genetic engineering techniques to the soluble ligands, and measured the neutralization activity. Thus, we confirmed that 5D12 (ATCC No. HB-11339) exhibits only slight neutralization activity.

[0011] We have newly found that 5D12, an antagonistic antibody, has agonistic activity on its own, as a result of cross-linking even in the absence of CD40L. Conventionally, it has been reported that the action of mouse CD40 antibodies is enhanced by cross-linking of biotin with avidin (Johnson et al., Eur J Immunol, 24: 1835, 1994). Furthermore, it has been known that solid-phasing of CD40 antibodies using anti-immunoglobulin antibodies solid-phased on a plate leads to an increase in activity to suppress the proliferation of tumor cells. This has been thought to be an effect resulting from solid-phasing. However, it has not been known that when anti-immunoglobulin antibodies are added to a culture solution for cross-linking of anti-CD40 antibodies, it may become possible even for antagonistic antibodies to show agonistic activity. If antibodies to be used for therapy have antigenicity, a completely opposite effect may occur, such that antibodies which bind to CD40 antibodies in a human body are produced, and with which CD40 antibodies are cross-linked, so that activity seemingly the same as that of CD40 ligands is produced. Accordingly, in view of the safety of a therapeutic agent, it is very important to keep the antigenicity of antibodies at a low level. Consider a case wherein a therapeutic agent is developed by humanization technology based on the sequence of a variable region of a mouse antibody. Since humanized antibodies are known to have immunogenicity, anti-humanized anti-CD40 antibodies may be produced after administration. Specifically, there may be a risk that the antibodies would become agonistic antibodies. Even if the antigenicity is low, anti-CD40 antibodies may be cross-linked with antibody receptors (FcR). From these points, a preferred antagonistic antibody is a human antibody, which binds specifically to CD40, suppresses the binding of CD40L, and does not activate CD40 even by cross-linking, and exhibits weak binding to FcR.

## SUMMARY OF THE INVENTION

[0012] As described above, the functions of DC have been increasingly analyzed recently, so that CD40 has begun to be recognized as a gene important in controlling the functions of DC. Starting from this background, the purpose of the present invention is to provide by employing an evaluation system using DC, an anti-human CD40 antibody or a functional fragment thereof, which is substantially antagonistic also to a human CD40 antigen on the dendritic cell (DC) surface, and an agonistic anti-human CD40 antibody or a functional fragment thereof that is expected to have a therapeutic effect higher than that of the conventional anti-human CD40 antibody.

[0013] As a result of intensive studies concerning the preparation of antibodies against human CD40, we have completed the present invention by succeeding in producing a novel agonistic antibody and antagonistic antibody that are thought to have a therapeutic effect against disease higher than that of the conventionally known anti-CD40 antibody. That is, the present invention is as follows.

(1) An antibody against a human CD40, or a functional fragment thereof, having at least one property selected from the following properties (a) to (f) of:

- (a) acting on dendritic cells to produce IL-12 in the presence of LPS and IFN $\gamma$ ;
- (b) having activity to act on dendritic cells causing the cells to mature, which is higher than that of a G28-5 antibody;
- (c) having activity to promote an established B cell line to express CD95, which is higher than that of the G28-5 antibody;
- (d) having activity to suppress the proliferation of an established B cell line, which is higher than that of the G28-5 antibody;

- (e) inducing cell death of an established B cell line; and  
 (f) not inhibiting the binding of CD40 ligands to CD40.

(2) The above antibody or the functional fragment thereof of the present invention, wherein the maturation of dendritic cells is performed at a concentration of 20 µg/ml or less. In addition, the antibody or the functional fragment thereof promote the established B cell line to express CD95 at the antibody concentration of 20 µg/ml or less. Examples of the established B cell line include Ramos, HS-Sulton or the like.

(3) Furthermore, the above antibody or the functional fragment thereof of the present invention leads to the production of 100 pg/ml or more IL-12 when the antibodies with a concentration of 0.1 µg/ml or more are added to dendritic cells with a concentration of  $1 \times 10^6$  cells/ml, and the production of 1000 pg/ml or more, preferably 10000 pg/ml or more IL-12 when the antibodies with a concentration of 1 µg/ml or more are added.

(4) Furthermore, within the antibody concentration range between 0.01 µg/ml and 10 µg/ml, the above antibody or the functional fragment thereof of the present invention, promoting the established B cell line (Ramos cell) to express CD95 with approximately 2 to 3 times or more greater effectiveness than that expressed by a G28-5 antibody as a control. For example, with an antibody concentration of 0.01 µg/ml, the expression is promoted with approximately 2 to 6 times or more greater effectiveness than that expressed by the G28-5 antibody as a control. With an antibody concentration of 0.1 µg/ml, the expression is promoted with approximately 2 to 7 times or more greater effectiveness than that expressed by the G28-5 antibody as a control. With an antibody concentration of 1 µg/ml, the expression is promoted with approximately 2 to 7 times or more greater effectiveness than that expressed by the G28-5 antibody as a control. With an antibody concentration of 10 µg/ml, the expression is promoted with approximately 2 to 6 times or more greater effectiveness than that expressed by the G28-5 antibody as a control.

(5) An antibody or a functional fragment thereof, having the amino acid sequences of a heavy chain variable region and a light chain variable region of an antibody that is produced by a hybridoma KM302-1 (Accession No: FERM BP-7578), KM341-1-19 (Accession No: FERM BP-7759), 2105 (Accession No: FERM BP-8024) or F1-102 (Accession No: ATCC PTA-3337).

Name	Accession No.	Deposition date	Deposited with:
KM302-1	FERM BP-7578	May 9, 2001	International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (Central 6, 1-1-1, Higashi, Tsukuba, Ibaraki, Japan)
KM341-1-19	FERM BP-7759	September 27, 2001	
2105	FERM BP-8024	April 17, 2002	
F1-102	ATCC PTA-3337	April 24, 2001	American Type Culture Collection (10801 University Blvd. Manassas, Virginia, 20110-2209, U.S.A.)

(6) An antibody or a functional fragment thereof, having amino acid sequences of the mature portions of a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma F2-103, which are respectively encoded by plasmid DNAs with Accession Nos. ATCC PTA-3302 and ATCC PTA-3303; a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma F5-77, which are respectively encoded by plasmid DNAs with Accession Nos. ATCC PTA-3304 and ATCC PTA-3305; or a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma F5-157, which are respectively encoded by plasmid DNAs with Accession Nos. ATCC PTA-3306 and ATCC PTA-3307.

Name	Accession No.	Deposition date	Deposited with:
F2-103 heavy chain (F2-103-H)	ATCC PTA-3302	April 19, 2001	American Type Culture Collection (10801 University Blvd. Manassas, Virginia, 20110-2209, U.S.A.)
F2-103 light chain (F2-103-L)	ATCC PTA-3303	April 19, 2001	
F5-77 heavy chain (F5-77-H)	ATCC PTA-3304	April 19, 2001	
F5-77 light chain (F5-77-L)	ATCC PTA-3305	April 19, 2001	
F5-157 heavy chain (F5-157-H)	ATCC PTA-3306	April 19, 2001	
F5-157 light chain (F5-157-L)	ATCC PTA-3307	April 19, 2001	

(7) An antibody or a functional fragment thereof, having amino acid sequences of the mature portions of a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma KM341-1-19, which

are respectively represented by SEQ ID NOS: 28 and 30; a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma 2105, which are respectively represented by SEQ ID NOS: 32 and 34; a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma 110, which are respectively represented by SEQ ID NOS: 36 and 38; a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma 115, which are respectively represented by SEQ ID NOS: 40 and 42; a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma KM643-4-11, which are respectively represented by SEQ ID NOS: 52 and 54; a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma F2-103, which are respectively represented by SEQ ID NOS: 60 and 62; or a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma F5-77, which are respectively represented by SEQ ID NOS: 64 and 66.

(8) An antibody or a functional fragment thereof, having amino acid sequences of the mature portions of a heavy chain variable region and a light chain variable region that are encoded by nucleic acid sequences isolated from a hybridoma KM341-1-19, which are respectively represented by SEQ ID NOS: 27 and 29; a heavy chain variable region and a light chain variable region that are encoded by nucleic acid sequences isolated from a hybridoma 2105, which are respectively represented by SEQ ID NOS: 31 and 33; a heavy chain variable region and a light chain variable region that are encoded by nucleic acid sequences isolated from a hybridoma 110, which are respectively represented by SEQ ID NOS: 35 and 37; a heavy chain variable region and a light chain variable region that are encoded by nucleic acid sequences isolated from a hybridoma 115, which are respectively represented by SEQ ID NOS: 39 and 41; a heavy chain variable region and a light chain variable region that are encoded by nucleic acid sequences isolated from a hybridoma KM643-4-11, which are respectively represented by SEQ ID NOS: 51 and 53; a heavy chain variable region and a light chain variable region that are encoded by nucleic acid sequences isolated from a hybridoma F2-103, which are respectively represented by SEQ ID NOS: 59 and 61; or a heavy chain variable region and a light chain variable region that are encoded by nucleic acid sequences isolated from a hybridoma F5-77, which are respectively represented by SEQ ID NOS: 63 and 65.

(9) An antibody against a human CD40, or a functional fragment thereof, having at least one property selected from the following properties (g) to (j) of:

(g) neutralizing the action of ligands on CD40;

(h) neutralizing or alleviating one or more effects that ligands, which are for CD40 on an established B cell line, have on CD40-expressing cells, and having agonistic action on CD40 on the above established B cell line weaker than that of 5D12 due to cross-linking by anti-immunoglobulin antibodies;

(i) alleviating or neutralizing the action of CD40 ligands on the established B cell line to increase CD95 expression; and

(j) having antagonistic action on CD40 expressed on dendritic cells.

(10) The antibody or the functional fragment of (9) above can suppress the expression of CD95 in Ramos cells to a level approximately 10% or less than that of a control, when antibodies with a concentration of 0.1 µg/ml are added to the Ramos cells with a concentration of  $1 \times 10^6$  cells/ml supplemented with a saturated amount of CD40L-expressing cells; can suppress the expression of CD95 in Ramos cells to the same level as that of a negative control, when the antibodies with a concentration of 1 µg/ml are added; and can suppress the expression of CD95 in the Ramos cells to the same level as that of the negative control, when the antibodies with a concentration of 10 µg/ml are added.

(11) The antibody or the functional fragment thereof of (9) above, wherein the proliferation of tonsillar B cells is suppressed in vitro by approximately 80 to 95% or more, when the antibodies with a concentration between 0.001 µg/ml and 10 µg/ml are added to  $1 \times 10^5$  tonsillar B cells supplemented with soluble CD40L (1 µg/ml). For example, when the antibodies with a concentration between 0.01 µg/ml and 10 µg/ml are added, the proliferation of tonsillar B cells is suppressed by approximately 95% or more. In particular, when the antibodies with a concentration of 0.001 µg/ml are added, the proliferation of tonsillar B cells is suppressed by approximately 80% or more.

(12) An antibody or a functional fragment thereof, having amino acid sequences of a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma KM281-1-10 (Accession No: FERM BP-7579), 4D11 (Accession No: FERM BP-7758) or F4-465 (Accession No: ATCC PTA-3338).

Name	Accession No.	Deposition date	Deposited with:
KM281-1-10	FERM BP-7579	May 9, 2001	International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (Central 6, 1-1-1, Higashi, Tsukuba, Ibaraki, Japan)
4D11	FERM BP-7758	September 27, 2001	

(continued)

Name	Accession No.	Deposition date	Deposited with:
F4-465	ATCC PTA-3338	April 24, 2001	American Type Culture Collection (10801 University Blvd. Manassas, VA 20110-2209, U.S.A.)

(13) An antibody or a functional fragment thereof, having amino acid sequences of the mature portions of a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma KM281-1-10, which are respectively represented by SEQ ID NOS: 44 and 46; a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma 4D11, which are respectively represented by SEQ ID NOS: 48 and 50; or a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma F4-465, which are respectively represented by SEQ ID NOS: 56 and 58.

(14) An antibody or a functional fragment thereof, having amino acid sequences of the mature portions of a heavy chain variable region and a light chain variable region that are encoded by nucleic acid sequences isolated from a hybridoma KM281-1-10, which are respectively represented by SEQ ID NOS: 43 and 45; a heavy chain variable region and a light chain variable region of an antibody produced by a hybridoma 4D11, which are respectively represented by SEQ ID NOS: 47 and 49; or a heavy chain variable region and a light chain variable region that are encoded by nucleic acid sequences isolated from a hybridoma F4-465, which are respectively represented by SEQ ID NOS: 55 and 57.

(15) Examples of the antibody or the functional fragment thereof of (1) to (14) above include human antibodies.

(16) A pharmaceutical composition, containing as an active ingredient the antibody or the functional fragment thereof of any one of (1) to (15) above.

(17) An immunopotentiating agent, anti-tumor agent or anti-autoimmune disease agent, containing as an active ingredient the antibody or the functional fragment thereof of any one of (1) to (8) above.

(18) An immunosuppressive agent, anti-autoimmune disease agent, therapeutic agent against allergies or therapeutic agent against blood coagulation factor VIII-inhibiting syndrome, containing as an active ingredient the antibody or the functional fragment thereof of any one of (9) to (14) above.

(19) Here, an epitope of a human CD40 that the monoclonal antibody of the present invention recognizes can be determined by a known method, such as by examining the binding to overlapping synthetic oligopeptides obtained from the primary amino acid sequence of human CD40 (e.g., Ed Harlow and David Lane (eds.), Antibodies: A Laboratory Manual, 1988 Cold Spring Harbor Laboratory Press; US Patent No. 4708871). A peptide library kit with the phage display process (New England BioLabs) can also be used for epitope mapping. The present invention also encompasses an antibody or a functional fragment thereof that recognizes a novel epitope of human CD40 that the antibody or the functional fragment thereof produced by each of the above hybridomas recognizes.

(20) The present invention further provides a nucleic acid (RNA or cDNA) encoding at least the variable region of a heavy chain and/or light chain of an antibody isolated from each of the above hybridomas, a vector containing the nucleic acid, and a host cell carrying the nucleic acid.

**[0014]** The present invention will be described in detail. This specification includes part or all of the contents as disclosed in the specification and/or drawings of PCT Application PCT/US01/13672 (filed on April 27, 2001), Japanese Patent Application No. 2001-142482 (filed on May 11, 2001), Japanese Patent Application No. 2001-310535 (filed on October 5, 2001), and U.S. Patent Application USSN 10/040,244 (filed on October 26, 2001) which are priority documents of the present application.

**[0015]** As described later, we have found that a known monoclonal antibody 5D12 (ATCC No. HB-11339) that is antagonistic to CD40 on B cells is not antagonistic to CD40 on DC. We have further found that many monoclonal antibodies show agonistic activity on their own as a result of cross-linking by anti-immunoglobulin antibodies, even if they are antagonistic antibodies that block the action of CD40L.

## 1. Definition

**[0016]** The terms used in this specification are defined as follows.

**[0017]** The term "human CD40" in the present invention means a polypeptide having an amino acid sequence shown by Clark et al. (E. A. Clark et al., Proc. Natl. Acad. Sci. USA 83: 4494, 1986) or Stamenkovic et al. (I. Stamenkovic et al., EMBO J. 8: 1403, 1989). Specifically, the human CD40 is an antigen polypeptide that is expressed on the surface of a B cell, DC, macrophage, endothelial cell, epithelial cell or tumor cells of these cells.

**[0018]** The term "anti-CD40 monoclonal antibody" means any monoclonal antibody against CD40 expressed by a cell, full-length CD40 or partial length CD40. A more preferred anti-CD40 monoclonal antibody binds to the extracellular

portion of CD40 and provides agonistic or antagonistic action on the cells expressing CD40.

[0019] Furthermore, the term "antibody" in the present invention is derived from a gene (generically called an "antibody gene") encoding a heavy chain variable region, a heavy chain constant region, a light chain variable region and a light chain constant region composing an immunoglobulin. The antibody of the present invention encompasses an antibody that is of any immunoglobulin class and has any isotype. The term "functional fragment" of the antibody in the present invention is a part (a partial fragment) of an antibody as defined above, and means a fragment retaining one or more actions of the antibody on an antigen. Specific examples of such functional fragment include F(ab')<sub>2</sub>, Fab', Fab, Fv, FVs with disulfide bond, single-stranded FV(scFV), and polymers thereof (D. J. King., Applications and Engineering of Monoclonal Antibodies., 1998 T. J. International Ltd).

[0020] The term "human antibody" in the present invention means an antibody which is the expression product of a human-derived antibody gene.

[0021] The term "agonistic" means an action to promote the binding of CD40 ligands to CD40 expressed on the surfaces of cells such as B cells, tumor cells or dendritic cells, or an action to provide CD40-expressing cells with one or more effects that are provided by CD40 ligands to CD40-expressing cells. The term "agonistic antibody" means an antibody having such an agonistic action.

[0022] The term "antagonistic" means an action to inhibit the binding of CD40 ligands to CD40 expressed on the surfaces of cells such as B cells, tumor cells or dendritic cells, or an action to neutralize one or more effects that are provided by CD40 ligands to CD40-expressing cells. The term "antagonistic antibody" means an antibody having such an action.

[0023] The term "dendritic cells (DC)" in the present invention indicates a group of cells which are also referred to as dendritic leukocytes having a strong antigen-presenting function. Dendritic cells used herein are induced by culturing CD34 positive precursor cells contained in, for example, bone marrow, umbilical cord blood or peripheral blood. Alternatively, the dendritic cells can be obtained by culturing CD14 positive monocytes in peripheral blood in the presence of GM-CSF and IL-4.

[0024] The term "immature DC" means DC that are CD14 negative, CD1a strongly positive, CD83, CD86 positive, and MHC class II positive.

[0025] The term "mature DC" means DC that are CD14 negative, CD1a positive, and have become CD83, CD86 and MHC class II strongly positive.

[0026] The term "activate DC" in the present invention means a change that DC induce by responding to the stimulation by CD40. For example, it also means to cause the maturation of immature DC, the high expression of CD80, CD86 and HLA-Class II, and the enhancement of IL-12 production. Alternatively, when T cells co-exist, it also means to stimulate T cells to promote their proliferation.

[0027] The term "activate B cells and a B cell line" in the present invention means a change that cells induce by responding to the stimulation by CD40. For example, it means to cause DNA synthesis, promote the incorporation of thymidine, and thus to increase the expression amount of CD95.

## 2. Obtainment of antibody

[0028] To obtain the antibody of the present invention, it is preferred to immunize mice using as an antigen a gene recombinant mouse cell line expressing a human CD40 or a soluble human CD40 that has been produced and purified with recombinants. Mice to be used for immunization are preferred to produce human antibodies (Tomizuka. et al., Proc Natl Acad Sci USA., 2000 Vol 97: 722). By selecting monoclonal antibodies that bind to soluble human CD40 that has been produced and purified with recombinants, antibodies that react also to CD40 expressed on cells other than B cells may be more easily obtained than by a case wherein clones reacting specifically to B cells are selected. Hybridomas can be produced by the method of Kohler and Milstein et al. (Nature, 1975 Vol. 256: 495) generally used in monoclonal antibody production using the lymphnode cells or splenocytes of immunized mice.

[0029] Furthermore, the binding of soluble CD40L to CD40 is analyzed using a surface plasmon resonance system such as BIAcore 2000 (Biacore), and then antibodies that do not compete with CD40L are selected. In addition, antibodies that suppress independently the suppression of the cell growth of B lymphoma are selected. Furthermore, antibody selection is performed using the condition of whether or not they act on DC as an indicator. This enables the production and selection of antibodies with advantages of acting on dendritic cells or B cells without competing with CD40L, and suppressing the proliferation of CD40-expressing cancer cells.

[0030] The antibody of the present invention is obtained by culturing the thus obtained hybridoma. Further, a gene encoding a human monoclonal antibody or a variable region thereof is cloned from an antibody-producing cell such as a B cell or a hybridoma, the cloned gene is incorporated into an appropriate vector, and then the vector is introduced into a host (e.g., a mammalian cell line, *Escherichia coli*, yeast cell, insect cell or plant cell), so that a recombinant antibody produced by gene recombination technology can be prepared (P. J. Delves., ANTIBODY PRODUCTION ESSENTIAL TECHNIQUES., 1997 WILEY, P. Shepherd and C. Dean., Monoclonal Antibodies., 2000 OXFORD UNI-



VERSITY PRESS; J. W. Goding., Monoclonal Antibodies: principles and practice., 1993 ACADEMIC PRESS). Moreover, transgenic cattle, goat, sheep or pigs, wherein a target antibody gene is incorporated into the endogenous gene by transgenic animal generation techniques are generated. From the milk of these transgenic animals, monoclonal antibodies derived from the antibody gene can be obtained in large quantities. When hybridomas are cultured in vitro, they are grown, maintained and stored in a way suitable for various conditions such as the properties of cell species to be cultured, purposes of experiments and studies, and culturing methods. Then, hybridomas can be cultured using any nutrient medium that is induced and prepared from a known nutrient medium or known basic medium that is used for the production of monoclonal antibodies in the culture supernatant.

### 3. Screening

**[0031]** Screening for agonistic antibodies is performed by analysis using human B lymphoma, so that antibodies that promote CD95 expression can be selected. Antibodies are further added to a purified DC culture solution, and then antibodies causing maturation are selected. Alternatively, antibodies showing activity to proliferate T cells in a mixed-lymphocyte reaction using immature DC are selected. Furthermore, antibodies are added to mature DC, and then antibodies having action to promote IL-12 production are selected. Furthermore, antibodies having activity to suppress the growth of tumor cells expressing CD40 or activity to induce cell death of the tumor cells are selected. Competition with CD40L can be distinguished from other cases based on whether or not the antibody inhibits the binding of soluble CD40 with soluble CD40 ligands using, for example, a surface plasmon resonance system (BIOCore). Alternatively, it can also be distinguished from other cases based on whether or not the antibody enhances the action of CD40 ligands on a B cell line.

**[0032]** Screening for antagonistic antibodies is performed by analysis using human B lymphoma. Further addition of soluble CD40L having FLAG as a tag in the presence of anti-FLAG antibody enables screening for antibodies that inhibit more strongly the binding of soluble CD40L to CD40 on the human B lymphoma cell. By the introduction of a gene encoding CD40L instead of soluble CD40L, it is also possible to use recombinant cells expressing many CD40 ligands on the cell surface. Subsequently, human antibodies are cross-linked with anti-human IgG antibodies, so that clones that activate B lymphoma by cross-linking are removed. Furthermore, antibodies showing activity to suppress the T cell proliferation in a mixed-lymphocyte reaction using purified and matured DC, or antibodies having action to suppress IL-12 production when CD40 ligands are added to mature DC are selected.

**[0033]** Antibodies that are obtained as described above have at least any of the following properties that are thought to be therapeutically effective.

#### (1) In the case of agonistic antibody

##### **[0034]**

(a) The antibody acts on dendritic cells to cause IL-12 production in the presence of LPS (lipopolysaccharide) and IFN $\gamma$ . The LPS concentration in this case ranges from 10 pg/ml to 10 ng/ml and the IFN $\gamma$  concentration ranges from  $10^{-4}$  M to  $10^{-2}$  M. With an antibody concentration of 1  $\mu$ g/ml or more, or preferably, 0.1  $\mu$ g/ml or more, the production amount of IL-12 is greater than that in a test using a G28-5 antibody as a control, the known agonistic anti-CD40 antibody. When the antibodies with a concentration of 0.1  $\mu$ g/ml or more are added to dendritic cells with a concentration of  $1 \times 10^6$  cells/ml, 100 pg/ml or more IL-12 is produced, or when the same with a concentration of 1  $\mu$ g/ml or more are added, 1,000 pg/ml or more, or preferably, 10,000 pg/ml or more IL-12 is produced (see Examples 9 and 13).

(b) The antibody has action of binding to dendritic cells and thus to cause the maturation of the dendritic cells. Moreover, when the antibodies with a concentration of 20  $\mu$ g/ml or less, preferably 0.1 to 15  $\mu$ g/ml; further preferably 5 to 15  $\mu$ g/ml were cultured with dendritic cells, the activity to cause maturation is higher than that of the G28-5 antibody (see Example 9).

(c) The antibody has activity to promote CD95 expression of an established B cell line, which is greater than that of the G28-5 antibody. In this case, with an antibody concentration of 10  $\mu$ g/ml or more, preferably 1  $\mu$ g/ml or more, further preferably 0.1  $\mu$ g/ml or more, still further preferably 0.01  $\mu$ g/ml or more, and most preferably 0.001  $\mu$ g/ml or more, the activity to promote CD95 expression is higher than that of the G28-5 antibody that is used as a control in a test. The ratios of the activity of the G28-5 antibody, which was used in a test as a control, to promote CD95 expression to the same of the antibody with concentrations of 10  $\mu$ g/ml, 1  $\mu$ g/ml, 0.1  $\mu$ g/ml and 0.01  $\mu$ g/ml are as shown below (Table 1).

Table 1

Antibody concentration	Ratio
10 µg/ml	Approximately 2-fold, preferably approximately 3-fold, more preferably 4.5-fold, and further preferably 6-fold
1 µg/ml	Approximately 2-fold, preferably approximately 5-fold, more preferably approximately 6-fold, and further more preferably 7-fold
0.1 µg/ml	2-fold, preferably 6-fold, more preferably approximately 7-fold
0.01 µg/ml	2-fold, preferably 4-fold, more preferably 5-fold, further preferably approximately 6-fold

The promoted expression of CD95 means that the antibody activates the established B cell line. Here, examples of the established B cell line include Ramos cells and HS-Sulton cells. In addition, Ramos cells are of Burkitt's lymphoma, which are model cells of human centroblastic B cells. HS-Sulton cells are of Burkitt's lymphoma (see Examples 6 and 12).

(d) The antibody has activity to suppress the DNA synthesis, thymidine incorporation, and proliferation of the established B cell line (Ramos cells or HS-Sulton cells), which is higher than that of G28-5 antibody. The antibody concentration in this case is at least 0.05 µg/ml, or preferably 0.1 to 15 µg/ml (see Example 8).

(e) The antibody induces cell death of the established B cell line (see Example 16).

(f) The antibody does not inhibit the binding of CD40 ligands to CD40. The term "does not inhibit" means that CD40L can bind to CD40 to the same degree as that when the antibody is absent, even when the antibody previously binds to CD40 (that is, in the presence of the antibody). Either one of or both CD40 ligands and CD40 may be a type of a protein that is expressed on the membrane or a soluble protein (see Example 11).

**[0035]** Antibodies having the above properties are produced, for example, by a hybridoma KM302-1 (FERM BP-7578) and a hybridoma KM341-1-19 (FERM BP-7759).

**[0036]** The nucleotide sequences and amino acid sequences of the heavy chain (H chain) and light chain (L chain) variable regions of a monoclonal antibody produced by the hybridoma KM341-1-19 were determined (Example 17). The present invention provides DNA encoding at least the heavy chain variable region, or the full-length heavy chain, and DNA encoding the light chain variable region of the monoclonal antibody produced by the hybridoma KM341-1-19. The DNAs also include other DNAs encoding the same amino acid sequences due to codon degeneration in addition to those described in Example 17. Moreover, the present invention provides monoclonal antibodies or functional fragments thereof as specified by the amino acid sequences of at least the heavy chain variable regions or the amino acid sequences of the full-length heavy chains, and the amino acid sequences of the light chain variable regions, as disclosed in Example 17.

(2) In the case of antagonistic antibody

**[0037]**

(g) The antibody neutralizes the action of ligands for CD40. Here, the term "the action of ligands" means both the action of ligands expressed on T cells or other cells, and the action of free ligands for CD40 (see Examples 7 and 14).

(h) The antibody neutralizes one or more effects that ligands for CD40 on the established B cell line have on CD40-expressing cells, and do not show agonistic action to CD40 on the above established B cell line by cross-linking by anti-immunoglobulin antibodies. This action is weaker than that of 5D12. The "effects that ligands have on CD40-expressing cells" mean the activation of the CD40-expressing cells. Specifically in B cells, the effect means the activation of thymidine incorporation and B cell proliferation, and the activation of the enhanced expression of CD95 in the established B cell line. Furthermore, in DC, the effect means the activation of DC maturation, the activation of the enhanced expression of CD86 and HLA-DR, the activation of thymidine incorporation by the co-existing T cells, the promotion of the proliferation, the activation of IL-12 and IL-10 production, and the like. Cross-linking by anti-immunoglobulin antibodies is performed by causing the presence of 0.1 µg/ml or more of anti-immunoglobulin antibodies in a culture solution (see Example 7).

(i) The antibody alleviates or neutralizes the activity of cross-linked CD40L or CD40L expressed by cells to enhance the expression of CD95 in the established B cell line. The antibody also alleviates or neutralizes the activity of CD40L, the action of which is enhanced by cross-linking by antibodies and the like against tags. The binding of

ligands (including both free ligands and ligands expressed by specific cells) for CD40 to CD40-expressing cells causes intracellular signal transduction, and finally causes the cells to express CD95 (Fas) on the cell surfaces. Accordingly, the antagonistic antibody of the present invention inhibits the above signal transduction by binding to CD40, thereby neutralizing the expression of CD95. The antibody concentration in this case is 1 µg/ml or more, or preferably 0.1 µg/ml or more (see Examples 7 and 14).

(j) The antibody is antagonistic to CD40 on DC. Specifically, the antibody alleviates or neutralizes the activity of CD40L to activate DC. When DC are stimulated by ligands on T cells co-existing with the DC, T cells are activated, so that thymidine incorporation and the like are promoted. In the mixed-lymphocyte reaction, wherein DC and T cells that are both derived from different individuals are allowed to co-exist, DC interact with T cells, thereby causing T-cell activation. The antagonistic antibody of the present invention inhibits the above interaction by binding to CD40, resulting in suppressed incorporation of thymidine. The antibody concentration in this case is at least 0.001 µg/ml, or preferably 0.1 to 10 µg/ml (see Example 10).

**[0038]** The above antagonistic antibody is produced by, for example, hybridomas KM281-1-10 (FERM BP-7579) and KM281-2-10-1-2 (FERM BP-7580) (May 9, 2001, the International Patent Organism Depository (IPOD) at the National Institute of Advanced Industrial Science and Technology (Central 6, 1-1-1, Higashi, Tsukuba, Ibaraki) and 4D11 (FERM BP-7758).

**[0039]** (3) The antibody of the present invention can be altered to an antibody of a different subclass (for example, see EP314161 publication), by modification by genetic engineering techniques known by a person skilled in the art, specifically by substituting a region that defines the subclass of an antibody heavy chain with a region that defines another subclass. A heavy chain variable region and the constant region of another subclass can be directly linked. For example, an alteration of the subclass of the antibody of the present invention to IgG2 or IgG4 makes it possible to lower the binding degree of the antibody to a Fc receptor. Specifically, *Nhe* I site (GCTAGC) is introduced into a human antibody heavy chain, EU index 118 (Ala), 119 (Ser) site according to Kabat et al (Sequence of Proteins of Immunological Interest, 5<sup>th</sup> Ed. Public Health Service, National Institute of Health, Bethesda, Md. (1991)). By digestion using the restriction enzyme, switching to another subclass, IgG, can be performed without altering the amino acid. Moreover, artificial alteration of the amino acid sequence of a constant region, or the binding of a constant region sequence having such an altered sequence with the variable region of the antibody of the present invention can lower the binding degree to a Fc receptor (Lund J., et al., J. Immunol. 1991 vol 147: 2657-2662), or can also increase or decrease CDC activity (Tao M., et al., J. Exp. Med. 1991 vol 1025-1028, Idusogie E E., et al., J. Immunol. 2001 vol 166: 2571-5). Furthermore, to avoid the action of ADCC, CDC or the like, only IgG2 or IgG4 subclass antibodies can be previously selected. In addition, the binding of a radionuclide, bacterial toxin, chemotherapeutant, prodrug or the like with the antibody of the present invention can further enhance the therapeutic effect against disease such as cancer.

#### 4. Pharmaceutical composition

**[0040]** A pharmaceutical composition containing a pharmaceutical preparation that is the purified antibody of the present invention is also encompassed by the scope of the present invention. Such a pharmaceutical composition preferably contains a physiologically acceptable diluent or carrier in addition to the antibody, or may be a mixture with other antibodies or other drugs, such as antibiotics. Examples of the appropriate carrier include, but are not limited to, a physiological saline solution, a phosphate buffered saline solution, a phosphate buffered saline glucose solution and a buffered physiological saline. Alternatively, the antibody is freeze-dried, and then used when necessary by adding the above buffered aqueous solution for reconstruction. Examples of the route of administration include an oral route and a parenteral route including intravenous, intramuscular, hypodermic and intraperitoneal injections or drug delivery.

**[0041]** In this case, the effective dose to be administered as a combination of the effective dose of the antibody of the present invention, an appropriate diluent, and a pharmacologically acceptable carrier ranges from 0.1 mg to 100 mg per kg of body weight per administration. Administration is performed at intervals of 2 days to 8 weeks.

**[0042]** When a pharmaceutical composition containing the antibody of the present invention is used, and particularly, when the agonistic antibody is used, the composition is used as an immunopotentiating drug (anti-viral agent and anti-infective drug), anti-tumor agent or anti-autoimmune disease agent. Multiple examples of these diseases may occur together. Alternatively, the antibody can also be used as an adjuvant in combination with a vaccine such as a cancer-specific peptide. When the composition contains the antagonistic antibody, it is useful as an immunosuppressive agent (prophylactic or therapeutic agent against immunological rejection or GVHD upon transplantation of islets of Langerhans, kidneys or the like) upon organ transplantation, or an anti-autoimmune disease agent (e.g., against rheumatism, or as a therapeutic agent against arterial sclerosis, disseminated sclerosis, systemic erythematodes, idiopathic thrombocytopenia or Crohn's disease), therapeutic agent against allergies such as asthma, or therapeutic agent against blood coagulation factor VIII-inhibiting syndrome. Multiple examples of these diseases may occur together.

**[0043]** When the anti-CD40 antibody is used as a therapeutic means against a disease in which CD40 is involved,

it can be expected that antibodies providing a better therapeutic effect can be obtained by selecting the antibodies using the function of DC as an indicator.

[0044] In the case of the agonistic antibody, it can be expected that antibodies having strong immunopotential action can be obtained by selecting antibodies that can activate DC more effectively. Furthermore, by using the promotion of IL-12 production by mature DC as an indicator, antibodies having strong CTL-inducing action can be obtained. By the CTL induction, antibodies that are highly effective for removing cells infected with viruses or tumor cells can be obtained. Moreover, since synergistic effects can be expected, preferred antibodies bind to CD40 without inhibiting the binding of CD40 ligands to CD40. When cancer treatment is considered, if antibodies that directly induce cell death of CD40-expressing cancer cells or suppress their proliferation, and effectively activate DC are present, synergistic effects are expected therefrom, and such antibodies can be a therapeutic agent that can be used against tumors that do not express CD40. These antibodies are considered to be useful as a therapeutic agents against viral diseases or anti-tumor agents.

[0045] In the meantime, antibodies that specifically bind to CD40 and suppress the binding of CD40L without activating CD40 are also expected to be able to suppress not only the action of ligands for B cells, but also the action on DC. However, antibodies have been so far obtained using as an indicator their effect on B cells. Thus, it is highly significant to obtain antibodies that have strong suppressive action also on dendritic cells and to develop them as a pharmaceutical product. Further, it is a concern that the anti-CD40 antibody can have a totally opposite action by cross-linking as described above. Thus, antibodies that do not activate CD40 even by cross-linking are required. It is also a concern that monoclonal antibodies derived from a non-human mammal such as a mouse, chimeric antibodies consisting of the variable region of a mouse monoclonal antibody and a constant region of a human immunoglobulin and humanized antibodies resulting from CDR grafting, which have been so far reported as antibodies against human CD40, have antigenicity. Therefore, a human antibody is desirable as an antibody to inhibit the binding with CD40 ligands.

#### BRIEF DESCRIPTION OF DRAWINGS

##### [0046]

Fig.1 shows that KM302-1 antibodies promoted CD95 expression.

Fig.2A shows that the antagonistic antibodies neutralized the action of CD40 ligands on Ramos cells.

Fig.2B shows that the antagonistic antibodies neutralized the action of CD40 ligands on Ramos cells.

Fig.3 shows that KM281-1-10 antibodies neutralized the action of CD40 ligands on Ramos cells.

Fig.4 shows that cross-linked KM281-1-10 antibodies did not promote CD95 expression.

Fig.5 shows that cross-linked 5D12 antibodies promoted CD95 expression.

Fig.6 shows the proliferation suppressive effect of KM302-1 antibodies on tumor cells.

Fig.7 shows that KM302-1 antibodies promoted the maturation of DC.

Fig.8 shows that KM302-1 antibodies promoted the IL-12 production of DC.

Fig.9 shows that KM281-1-10 antibodies neutralized the action of CD40 ligands on DC.

Fig. 10 shows that KM281-1-10 antibodies neutralized the action of CD40 ligands on DC.

Fig. 11 shows that KM302-1 antibodies activated immature DC-MLR.

Fig.12 shows that KM341-1-19 antibodies and the like promoted CD95 expression of Ramos cells.

Fig.13 shows that KM341-1-19 antibodies promoted IL-12 production of mature DC.

Fig.14 shows that KM341-1-19 antibodies promoted IL-10 production of mature DC.

Fig.15 shows that 4D11 antibodies and the like neutralized the action of CD 40 ligands on Ramos cells.

Fig.16 shows that KM302-1 antibodies showed anti-tumor effect on the human tumor cell-transplanted mouse model.

Fig.17 shows that KM341-1-19 antibodies showed a proliferation suppressive effect against tumor cells.

Fig.18 shows that F4-465, 4D11 and KM281-1-10 suppressed antigen-specific IgG production.

Fig.19 shows that F4-465, 4D11 and KM281-1-10 suppressed antigen-specific IgM production.

Fig.20 shows that F4-465 suppressed the proliferation of tonsillar B cells.

#### BEST MODE FOR CARRYING OUT THE INVENTION

[0047] The present invention will be further described in detail by referring to the examples. However, the technical scope of the invention is not limited by these examples.

## [Example 1] Preparation of antigen

## (1) Cell

**[0048]** EL-4 cells are of a mouse-derived established T cell line, and can be easily obtained (ATCC No.: TIB-39). Ramos B cells (ATCC No.: CRL-1596) and mouse anti-CD40 antibody-producing hybridoma G28-5 (HB-9110) and 5D12 (HB-11339) were purchased from ATCC.

## (2) Expression and purification of antigen

**[0049]** Extracellular regions were amplified by PCR using human CD40 cDNA (Genbank Accession Number: NM\_001250) as a template and the following primers under conditions of 20 cycles of 95°C for 5 seconds, 55°C for 30 seconds and 72°C for 30 seconds.

**Primer 1:** 5'-CCCAGATCTGTCCATCCAGAACCACCCACTGCATGCAGAG-3'

(SEQ ID NO: 1)

**Primer 2:** 5'-ACAAGATCTGGGCTCTACGTATCTCAGCCGATCCTGGGGAC-3'

(SEQ ID NO: 2)

**[0050]** The amplified cDNA was inserted following the melittin signal sequence and before the human IgG1-derived FC or mouse IgG2a-derived FC region of a pFastBac vector (Gibco BRL). To produce CD40, recombinant baculoviruses were prepared according to the instruction. Th5 cells were infected with the recombinant viruses, and then cultured for 4 days. The supernatant was treated with a 0.22 nm filter, ProteinG sepharose (Amersham Pharmacia) was added thereto, and then the mixture was gently shaken at 4°C. After one night, sepharose was transferred to a column and then washed with a 20× volume of PBS. A human CD40 FC protein was eluted with a 20 mM glycine buffer (pH 3.0). The vector to express CD40 on cell surfaces was obtained from Randolph J. Noelle (Inui, S et al., EJI, 20, 1747-1753, 1990). The full-length cDNA was cleaved with the Xba I enzyme, and then inserted into pCDNA3 (INVITROGEN). The vector was introduced into EL-4 cells, and then the cells were cultured in the presence of 0.5 mg/ml G418 (Gibco BRL), thereby obtaining a stable expression strain. The expression of CD40 was confirmed by FACS analysis using FITC-conjugated anti-human CD40 antibodies (Pharmingen).

## [Example 2] Generation of mice for immunization

**[0051]** The mice used for immunization had a genetic background whereby they were homozygotes for both disrupted endogenous Ig heavy chain and κ light chain, and the mice harbored at the same time chromosome 14 fragment (SC20) containing human Ig heavy chain gene locus, and human Igκ chain transgene (KCo5). These mice were generated by crossing mice of a line A having a human Ig heavy chain gene locus with mice of a line B having a human Igκ chain transgene. The mice of line A are homozygous for both disrupted endogenous Ig heavy chain and κ light chain, and harbor chromosome 14 fragment (SC20), which is transmittable to progeny, as is described, for example, in the report of Tomizuka et al. (Tomizuka, et al., Proc Natl Acad Sci USA., 2000 Vol 97: 722). The mice of the line A were immunized, so that the following hybridomas F2-103 and F5-77 were obtained. Furthermore, the mice of line B (transgenic mice) are homozygotes for both disrupted endogenous Ig heavy chain and κ light chain, and harbor a human Igκ chain transgene (KCo5), as described, for example, in the report of Fishwild et al. (Nat Biotechnol., 1996 Vol 14:845).

**[0052]** Individuals obtained by crossing male mice of the line A with female mice of the line B, or female mice of the line A with male mice of the line B, and having human Ig heavy chain and κ light chain detected simultaneously in the sera (Ishida & Lonberg, IBC's 11th Antibody Engineering, Abstract 2000) were used for the following immunization experiment. In addition, the above human antibody-producing mice are available from Kirin Brewery Co., Ltd via contract. By immunizing the above mice, the following hybridomas KM302-1, KM341-1-19, KM643-4-11, 2053, 2105, 3821, 3822, 285, 110, 115, KM281-1-10, KM281-2-10-1-2, KM283-5, KM292-1-24, KM225-2-56, KM341-6-9, 4D11, 5H10, 11E1, 5G3, 3811, 3411 and 3417 were obtained. Moreover, chimeric mice (Kuroiwa et al., Nat Biotechnol., 2000 vol 18:1086) harboring human antibody Lambda chain reported by Kuroiwa et al. were also used for the following immunization experiment. A hybridoma F4-465 was obtained from the mouse.

## [Example 3] Preparation of human monoclonal antibody against human CD40

**[0053]** Monoclonal antibodies in this example were prepared according to a general method described in the Introduction of Experimental Procedures for Monoclonal Antibodies (written by Tamie ANDO et al., KODANSHA, 1991). The human CD40 used as an immunogen herein were the human CD40 human FC and CD40-expressing EL-4 cells prepared in Example 1. Animals used herein for immunization were human antibody-producing mice that produce the human immunoglobulin prepared in Example 2.

**[0054]** The human antibody-producing mice were immunized with 2 to 100 µg/immunization of CD40: hFc per mouse. Excluding the first immunization, an antigen solution was mixed with an equivalent volume of Freund's incomplete adjuvant (Sigma), and then injected subcutaneously into several separate positions. Immunization was performed 3 to 4 times approximately every 10 days to 3 weeks. For the first immunization, Freund's incomplete adjuvant (Sigma) was used. Blood was collected from the mouse tail, and then human antibody γ and κ against CD40 in the serum were measured using ELISA. 3 to 4 days before excision of the spleen, final immunization was performed by injecting 20 µg of CD40: Fc dissolved in PBS via the caudal vein.

**[0055]** The human antibody-producing mice were immunized with human CD40-expressing mouse EL-4 cells. EL-4 cells (10<sup>8</sup> cells/ml) were suspended in PBS, and then gently mixed with an equivalent volume of RIBI adjuvant previously emulsified with PBS. Immunization was performed with the cells 3 to 5 times approximately every 10 days to 3 weeks. When the adjuvant was not used, the cells were irradiated with X-rays with 8000 rad for use.

**[0056]** The spleen was surgically obtained from the immunized mice. The collected splenocytes were mixed with mouse myeloma SP2/0 (ATCC No.: CRL1581) at a ratio of 5 to 1. The cells were fused using polyethylene glycol 1500 (Boehringer Mannheim) as an agent for cell fusion, thereby preparing a large number of hybridomas. The selection of hybridomas was performed by culturing in HAT-containing DMEM media (Gibco BRL) supplemented with 10% fetal calf serum (FCS), hypoxanthine (H), aminopterin (A) and thymidine (T). Furthermore, single clones were obtained by the limiting dilution method using HT-containing DMEM media. Culturing was performed in a 96-well microtiter plate (Beckton Dickinson). Screening for hybridoma clones producing anti-human CD40 human monoclonal antibodies was performed by measurement using enzyme-linked immuno adsorbent assay (ELISA) and fluorescence activated cell sorter (FACS), as described later in Example 4.

**[0057]** Screening for the human monoclonal antibody-producing hybridoma by ELISA was performed by 3 types of ELISA and FACS analyses as described below. Thus, a large number of hybridomas producing human monoclonal antibodies that had human immunoglobulin γ chain (hIgγ) and human immunoglobulin light chain κ, and had reactivity specific to human CD40 were obtained. In any of the following examples including this example, and tables and figures showing the test results of the examples, each hybridoma clone producing the human anti-human CD40 monoclonal antibody of the present invention was denoted using symbols. A clone represented by the symbols followed by "antibody" means an antibody that is produced by each of the hybridomas, or a recombinant antibody that is produced by a host cell carrying an antibody gene (full-length or a variable region) isolated from the hybridoma. In addition, within a contextually clear range, the name of a hybridoma clone may express the name of an antibody.

**[0058]** The following hybridoma clones represent single clones.

## Agonistic antibody:

KM302-1, KM341-1-19, KM643-4-11, 2053, 2105, 3821, 3822, 285, 110, 115, F1-102, F2-103, F5-77 and F5-157

## Antagonistic antibody:

KM281-1-10, KM281-2-10-1-2, KM283-5, KM292-1-24, KM225-2-56, KM341-6-9, 4D11, 5H10, 11E1, 5G3, 3811, 3411, 3417 and F4-465

**[0059]** 3 hybridoma clones KM 302-1, KM 281-1-10 and KM 281-2-10-1-2 among them were deposited on May 9, 2001, clones KM341-1-19 and 4D11 were deposited on September 27, 2001, and clone 2105 was deposited on April 17, 2002, with the International Patent Organism Depositary at the National Institute of Advanced Industrial Science and Technology (Central 6, 1-1-1, Higashi, Tsukuba, Ibaraki, Japan) under the Budapest Treaty. Plasmids having the heavy chain and light chain variable regions of F2-103, F5-77 and F5-157, were deposited on April 19, 2001, and hybridoma clones F1-102 and F4-465 were deposited on April 24, 2001, with ATCC (American Type Culture Collection, 10801 University Blvd., Manassas, Virginia, U.S.A.) under the Budapest Treaty (Table 2).

Table 2

Name	Accession No.
KM302-1	FERM BP-7578
KM281-1-10	FERM BP-7579
KM281-2-10-1-2	FERM BP-7580
KM341-1-19	FERM BP-7759
4D11	FERM BP-7758
2105	FERM BP-8024
F1-102	ATCC PTA-3337
F4-465	ATCC PTA-3338
F2-103 heavy chain (F2-103-H)	ATCC PTA-3302
F2-103 light chain (F2-103-L)	ATCC PTA-3303
F5-77 heavy chain (F5-77-H)	ATCC PTA-3304
F5-77 light chain (F5-77-L)	ATCC PTA-3305
F5-157 heavy chain (F5-157-H)	ATCC PTA-3306
F5-157 light chain (F5-157-L)	ATCC PTA-3307

## [Example 4] Screening for hybridoma

Detection of monoclonal antibody having human immunoglobulin  $\gamma$  chain

**[0060]** The human CD40 mouse FC (1  $\mu$ g/ml) prepared in Example 1 was added at 50  $\mu$ l/well to each well of a 96-well microplate for ELISA (Maxisorp, Nunc) and incubated at 4°C for the human CD40 mouse FC to be adsorbed to the microplate. Next, the supernatant was discarded, and then a blocking reagent (Block Ace, DAINIPPON PHARMACEUTICAL) was added to each well, followed by incubation at room temperature for blocking. The culture supernatant (50  $\mu$ l) of each hybridoma was added to each well for reaction, and then each well was washed with a 0.1% Tween20-containing phosphate buffer (PBS-T). Goat anti-human IgG ( $\gamma$ ) antibody (Sigma, A0170) labeled with peroxidase was then diluted 5,000-fold with 1% FBS-containing PBS-T. The solution was added (50  $\mu$ l/well) to each well, and then incubation was performed. The microplate was washed 3 times with PBS-T, and then a chromogenic substrate solution (TMB, 50  $\mu$ l/well, SUMITOMO BAKELITE) was added to each well, followed by incubation at room temperature for 30 minutes. A stop solution was added (50  $\mu$ l/well) to each well to stop reaction. Absorbance at a wavelength of 450 nm was measured with a microplate reader. The culture supernatant of positive wells was analyzed by FACS, and then the wells wherein Ramos cells were stained were selected. The cells in the wells were cloned by the limiting dilution method, and then the cells of 1 clone were obtained per well. hx-positive status was confirmed by ELISA using the human CD40 mouse FC. As a result, anti-human CD40 antibodies of 173 clones were obtained from 20 mice. Some of these antibodies were shown in Table 3 (agonistic antibodies) and Table 4 (antagonistic antibodies). Among the agonistic antibodies, at least KM341-1-19 and 2105 did not significantly compete with ligands in a competitive test using CD40L-expressing cells, CD40-expressing cells and the antibodies.

Table 3

Agonistic antibody				
Hybridoma	Antigen	Subclass	DC	Tumor cell
KM302-1	CD40 mouse FC	IgG4	activated	suppressed proliferation
KM341-1-19	human CD40-expressing EL-4	IgG2	activated	suppressed proliferation
KM643-4-11	CD40 mouse FC	IgG1	not implemented	not implemented
2053	CD40 mouse FC	IgG2	not implemented	not implemented

Table 3 (continued)

Agonistic antibody				
Hybridoma	Antigen	Subclass	DC	Tumor cell
2105	CD40 mouse FC	IgG2	not implemented	not implemented
3821	human CD40-expressing EL-4	IgG3	not implemented	not implemented
3822	human CD40-expressing EL-4	IgG3	not implemented	not implemented
285	CD40 mouse FC	IgG 1	not implemented	not implemented
110	CD40 mouse FC	IgG4	not implemented	not implemented
115	CD40 mouse FC	IgG4	not implemented	not implemented
F2-103	CD40 mouse FC	IgG1	not implemented	not implemented
F5-77	CD40 mouse FC	IgG 1	not implemented	not implemented

Table 4

Antagonistic antibody				
Hybridoma	Antigen	Subclass	Effect of cross-linking	DC-MLR
KM281-1-10	CD40 mouse FC	IgG1	low	suppressed
KM281-2-10-1-2	CD40 mouse FC	IgG1	low	not implemented
KM283-5	CD40 mouse FC	IgG4	significant	not suppressed
KM225-2-56	CD40 mouse FC	IgG4	significant	not implemented
KM292-1-24	CD40 mouse FC	IgG2	significant	not implemented
KM341-6-9	human CD4-expressing EL-4	IgG1	significant	not implemented
4D11	CD40 mouse FC	IgG1	low	not implemented
5H10	CD40 mouse FC	IgG1	low	not implemented
11E1	CD40 mouse FC	IgG1	low	not implemented
5G3	CD40 mouse FC	IgG2	significant	not implemented
3811	human CD40-expressing EL-4	IgG1	significant	not implemented
3411	human CD40-expressing EL-4	IgG2	significant	not implemented
3417	human CD40-expressing EL-4	IgG2	significant	not implemented
F4-465	human CD40-expressing EL-4	IgG 1	not implemented	not implemented

[0061] Monoclonal antibodies having human immunoglobulin light chain  $\kappa$  (Ig $\kappa$ ) were detected in a manner similar to the above described ELISA method for human immunoglobulin  $\gamma$  chain except that goat anti-human Ig $\kappa$  antibodies (diluted 1,000-fold, 50  $\mu$ l/well, Southern Biotechnology) labeled with peroxidase were used.

[0062] The subclass of each monoclonal antibody was identified in a manner similar to the above ELISA method for human immunoglobulin  $\gamma$  chain, except that a sheep anti-human IgG1 antibody, sheep anti-human IgG2 antibody, sheep anti-human IgG3 antibody or sheep anti-human IgG4 antibody (each diluted 2,000-fold, 50  $\mu$ l/well, The Binding Site) labeled with peroxidase, was used. Reaction test of each monoclonal antibody against human CD40-expressing cells

[0063] The reactivity of each monoclonal antibody against a Ramos cell line reported to express CD40 was studied by FACS analysis.

[0064] The Ramos cell line was suspended at a concentration of  $2 \times 10^6$ /ml in a staining buffer (SB) of 0.1% Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub> and 2% FCS-containing PBS. The cell suspension (100  $\mu$ l/well) was apportioned to a 96-well round bottom plate (Beckton Dickinson). The culture supernatant (50  $\mu$ l) of each hybridoma was added, and then incubation was performed at ice temperature for 30 minutes. Human IgG1 antibodies against human serum albumin were used as a negative control, and prepared at a concentration of 2  $\mu$ g/ml with a hybridoma culture medium. 50  $\mu$ l of the solution was added, and then



incubation was performed at ice temperature for 15 minutes. After washing with SB, 50  $\mu$ l of R-PE fluorescence-labeled anti-human antibody (Southern Biotechnology) diluted 250-fold was added, and then incubation was performed at ice temperature for 15 minutes. After washing twice with SB, the product was suspended in 300 to 500  $\mu$ l of a FACS buffer, and then the fluorescence intensity of each cell was measured by FACS (FACSort and FACScan, Beckton Dickinson).

As a result, antibodies having binding activity for the Ramos cell line were selected.

#### [Example 5] Preparation of each antibody

**[0065]** The culture supernatant containing monoclonal antibodies was prepared by the following method.

**[0066]** A G28-5 antibody-producing hybridoma was obtained from ATCC (ATCC No. HB-9110). Anti-CD40 antibody-producing hybridomas were acclimatized in eRDF media (Kyokutoseiyaku) containing bovine insulin (5  $\mu$ g/ml, Gibco BRL), human transferrin (5  $\mu$ g/ml, Gibco BRL), ethanolamine (0.01 mM, Sigma) and sodium selenite ( $2.5 \times 10^{-5}$  mM, Sigma). The hybridomas were cultured in a spinner flask. When the viable cell rate of the hybridomas reached 90%, the culture supernatant was collected. The collected supernatant was applied to a 10  $\mu$ m and 0.2  $\mu$ m filters (German Science) so as to eliminate miscellaneous debris such as hybridomas.

**[0067]** Anti-CD40 antibodies were purified from the above culture supernatant by the following method. The culture supernatant containing the anti-CD40 antibodies was subjected to affinity purification using a Hyper D Protein A column (NGK INSULATORS, LTD) or a Protein G column (for purifying mouse IgG1, Amersham Pharmacia Biotech) according to the attached instruction using PBS (-) as an adsorption buffer and 0.1 M sodium citrate buffer (pH 3) as an elution buffer. 1 M Tris-HCl (pH 8.0) or  $\text{Na}_2\text{HPO}_4$  solution was added to adjust the elution fraction to have a pH of around 7.2. The prepared antibody solution was substituted with PBS (-) using a dialysis membrane (10000 cut, Spectrum Laboratories) or SP column (Amersham Pharmacia Biotech), and then sterilization by filtration was performed using a membrane filter MILLEX-GV (MILLIPORE) with a pore size of 0.22  $\mu$ m. The concentration of the purified antibody was found by measuring absorbance at 280 nm and then calculating with 1 mg/ml at 1.45 OD.

#### [Example 6] Promotion of CD95 expression in Ramos cells by anti-CD40 agonistic antibody

**[0068]** A  $5.0 \times 10^5$  cells/ml Ramos cell suspension was inoculated at 100  $\mu$ l/well ( $5 \times 10^4$  cells per well) to a 96-well plate. The hybridoma culture supernatant or the purified antibody was diluted to 20  $\mu$ g/ml with a medium, and then the solution was added at a concentration of 100  $\mu$ l/well to a 96-well plate. After overnight culture, the cells were collected and then analyzed by FACScan or FACSort (Beckton Dickinson) using R-PE-labeled anti-CD95 antibodies (Pharmingen NJ). Figure 1 shows the result. The horizontal axes in Fig. 1 indicate the expression intensity of CD95. Addition of antibodies is indicated with a thick line, and non addition of antibodies is indicated with a thin line. It was shown that the KM302-1 antibodies promoted CD95 expression better than G28-5 antibodies, which were the known antibodies. That is, the KM302-1 antibody was shown to be more effectively agonistic.

#### [Example 7] Suppression of CD95 expression in Ramos cell by anti-CD40 antagonistic antibody

**[0069]** A  $1.0 \times 10^6$  cells/ml Ramos cell suspension was inoculated at 50  $\mu$ l/well to a 96-well plate. The hybridoma culture supernatant or the purified antibody was adjusted at 2  $\mu$ g/ml with a medium, and then added at 100  $\mu$ l/well to a 96-well plate. Soluble CD40 ligands (4  $\mu$ g/ml, ALEXIS CORPORATION) and anti-FLAG antibodies (4  $\mu$ g/ml, M2, Sigma) were added to media, and then the media were added at 50  $\mu$ l/well to the 96-well plate. After overnight culture, the cells were collected and then analyzed by FACS using R-PE-labeled anti-CD95 antibodies (Pharmingen NJ). Figures 2A and 2B, and 3 show the results. The horizontal axes in the figures indicate the expression intensity of CD95. CD95 expression was suppressed to the same degree as that of a negative control by the antibodies produced by each of the following hybridomas: KM281-1-10, KM281-2-10-1-2, KM283-5, KM292-1-24 and KM225-2-56.

**[0070]** In Fig. 3, KM281-1-10 antibodies (lower panel) suppressed CD95 expression more effectively than that the 5D12 antibodies (central panel), the known antibody, only slightly suppressed CD95 expression. Specifically, the KM281-1-10 antibody was shown to be more effectively antagonistic. Thus, the human monoclonal antibody was shown to be an antagonistic antibody.

#### Effect of cross-linking by anti-immunoglobulin antibody

**[0071]** A  $1.0 \times 10^6$  cells/ml Ramos cell suspension was inoculated at 50  $\mu$ l/well to a 96-well plate. The hybridoma culture supernatant or the purified antibody was adjusted to 2  $\mu$ g/ml with a medium, and then added at 100  $\mu$ l/well to a 96-well plate. Anti-human IgG antibodies (Sigma, 13382) or anti-mouse IgG antibodies (Biosource, AM13401) were added at 4  $\mu$ g/ml to media, and then the media were added at 50  $\mu$ l/well to a 96-well plate. After overnight culture, the cells were collected, and then analyzed by FACS using R-PE-labeled anti-CD95 antibodies (Pharmingen NJ). Figures

4 and 5 show the results. The horizontal axes in the figures indicate the expression intensity of CD95. CD95 expression was suppressed by the antibodies produced by each of the hybridomas KM281-1-10 and KM281-2-10-1-2. Conversely, CD95 expression was enhanced by the antibodies produced by each of the following hybridomas, 5D12, KM283-5, KM292-1-24 and KM225-2-56.

[Example 8] Proliferation suppression in Ramos cells by anti-CD40 agonistic antibody

[0072] A  $1.0 \times 10^5$  cells/ml Ramos and HS-Sulton cell suspension was inoculated at 100  $\mu$ l/well to a 96-well plate. A mixture of equivalent amount of the purified antibodies or soluble CD40 ligands, and anti-FLAG antibodies (M2) was added to media. After 2 days of culturing, 10  $\mu$ l of 100  $\mu$ Ci/ml  $^3$ H-Thymidine (Amersham Pharmacia) was added. After 18 hours, the culture product was harvested in a Printed Filtermat A (Wallac) using a Macro 96 Harvester (SKATRON), dried, and then immersed well in Betap;Scint (Wallac). After packaging, activity was measured using a 1205 BETA-PLATE liquid scintillation counter. Figure 6 shows the results. In the figure, the longitudinal axes indicate the amount of  $^3$ H thymidine incorporated by cells, and the horizontal axes indicate the concentration of the antibody or CD40L in the culture solution. When the KM302-1 antibodies were added to Ramos cells and HS-Sulton cells, the amount of thymidine incorporated was lower than the conventional G28-5 antibodies and CD40L. Thus, it was shown that the KM302-1 antibody is an agonistic antibody that can effectively suppress the proliferation of tumor cells.

[Example 9] Activation of dendritic cell by CD40 agonistic antibody

(1) Materials and methods

[0073] Recombinant human IL-4 was purchased from Genzyme techne. Anti-human CD14 MACS beads were purchased from Miltenyi Biotech GmbH. Lymphoprep was purchased from Nycomed Pharma AS. The medium used for culturing was RPMI1640 (Gibco BRL) supplemented with 10% heat inactivated FCS (Cell Culture Technologies), 10mM HEPES (Sigma), 55  $\mu$ M 2-mercaptoethanol (Gibco BRL) and streptomycin sulfate (MEIJI SEIKA KAISHA, LTD.), when DC were induced. The cells in a staining process were washed with PBS (Sigma) supplemented with 2% FCS (Cell Culture Technologies) and 0.02% Azaid. When the cells were frozen, Cell banker (Nippon Zenyaku Kogyo) was used.

(2) Induction of monocyte-derived DC

[0074] Mononuclear cells were prepared (PBMC) from peripheral blood by density gradient centrifugation using Lymphoprep. The cells were subjected to positive selection using anti-human CD14 MACS beads, so as to separate the cells into a CD14 positive fraction and negative fraction. Recombinant human GM-CSF (50 ng/ml) and recombinant human IL-4 (100 ng/ml) were added to the positive fraction, followed by culturing in RPMI1640 media supplemented with 10% FCS in a 6-well plate. At the start of culturing, the cells were cultured at a concentration of  $1 \times 10^6$ /ml (3 ml per well). During culturing, the media were exchanged once every 2 days. Medium exchange was performed by sampling 10% of the culture solution in a centrifugation tube, centrifuging the solution, removing the supernatant, suspending with a new culture solution (containing cytokine and the like at the above concentration) in a volume 2-fold greater than the sampled culture solution, and then returning the suspension to each well. On day 6 of culturing, the cells were collected, the cell number was calculated, and then the cells were suspended at a concentration of  $1 \times 10^6$ /ml in the above media. Anti-CD40 antibodies or the isotype controls thereof were added to the media, and then cultured for further 4 days in a 24-well plate. During this period, no culture exchange was performed (cell number per well of  $1 \times 10^6$  cells, and cell concentration of  $1 \times 10^6$ /ml).

(3) Cell staining and analysis by flow cytometer

[0075] For staining, anti-HLA-DR antibodies (isotype control: rat IgG2a), anti-CD86 antibodies (isotype control: rat IgG1) and anti-CD83 antibodies (isotype control: rat IgG2b) were used. First, the antibodies were added, and then incubation was performed at 4°C for 30 minutes. After 3 washings, analysis was performed using the FACS Calibur (Beckton Dickinson).

(4) Increase in IL-12 secretion ability of Mature DC

[0076] After immature DC were obtained as described above, LPS (400 pg/ml) and IFN $\gamma$  ( $10^{-3}$ M) were added, and then culturing was performed for 2 days, thereby obtaining mature DC. To the mature DC, 10  $\mu$ g/ml anti-CD40 antibodies or the isotype control was added. For the supernatant after 24 hours, IL-12 production was measured using ELISA (Pharmingen).

## (5) Results and Discussion

[0077] Figure 7 shows the effect of KM302-1 antibodies, the agonistic antibodies, on DC maturation, and Figure 8 shows the effect of KM302-1 antibodies on IL-12 production of mature DC. The degree of maturation was compared with G28-5 antibodies as a control. When the expression of CD86 and that of HLA-DR were examined, the expression was further elevated, that is, the degree of maturation was increased in the case of KM302-1 antibodies compared with the case of G28-5 antibodies. It was also shown that IL-12 secretion was increased by the treatment of mature DC with KM302-1 antibodies. Accordingly, it was shown that the KM302-1 antibodies acted as agonistic antibodies on DC.

## [Example 10] DC-MLR

[0078] Blood (peripheral blood) collected from a normal human was centrifuged at 2000 rpm for 10 minutes, and then the serum was absorbed. The blood cell fraction was re-suspended with PBS, and then gently placed on Ficoll (Amersham Pharmacia). Centrifugation was performed at 2000 rpm for 30 minutes, so that a PBMC portion in the intermediate layer was collected, washed twice with PBS, and then used for a certain cell separation process using MACS.

[0079] Monocyte separation for culturing DC was performed according to the attached instruction using MACS (Miltenyi Biotec GmbH). This is briefly explained as follows. 800  $\mu$ l of MACS Buffer and 200  $\mu$ l of MACS CD 14 (Miltenyi Biotec GmbH, 502-01) were added to PBMC ( $1 \times 10^8$ ), and then treated at 4°C for 15 minutes. The cells were adsorbed to a MACS LS column, and then washed. The cells adsorbed to the column were collected as monocytes. MACS HLA-DR (Miltenyi Biotec GmbH, 461-01) was added to the cells that were not adsorbed to the column. HLA-DR positive cells were removed with a BS column, thereby preparing a T cell fraction. The proportion of CD3 positive cells was measured by FACS, and then substantial number of T cells was calculated from the total cell number in the T cell fraction. The obtained monocytes were cultured in R0 media (PPMI medium supplemented with  $\beta$ -mercapto ethanol (Gibco) and HEPES (SIGMA)) containing 100 ng/ml IL-4 (R&D system), 50 ng/ml G-CSF (KIRIN) and 10% FCS (SIGMA) at a concentration of  $1 \times 10^6$  cells/ml in a 6-well culture plate. On day 5 after culturing, 10 ng/ml LPS (DIFCO) was added for the cells to differentiate into mature DC.

[0080] MLR was performed by mixing T cells and mature DC, which had been isolated from different humans. The cell ratio of T cells to DC was determined as 1:80, and number of T cells was determined as  $2 \times 10^5$  cells/well. First, antibodies were added to DC for reaction to proceed for 30 minutes. Subsequently, T cells were added, culturing was performed for 4 days, and then 10  $\mu$ l of 100  $\mu$ Ci/ml  $^3$ H-Thymidine (Amersham Pharmacia) was added. 14 hours later, the cells were harvested in Printed Filtermat A (Wallac) using a Macro 96 Harvester (SKATRON), dried, and then immersed well in Betap;Scint (Wallac). After packaging, activity was measured using a 1205 BETAPLATE liquid scintillation counter. MLR using immature DC was performed by mixing T cells with mature DC, which had been isolated from different humans. With a cell ratio of T cells to DC of 1:40, MLR was performed similarly. Figures 9 and 10 show the results. It was shown that the addition of KM281-1-10 antibodies lowered thymidine incorporation, and thus MLR could be suppressed. Furthermore, it was shown in Fig. 10 that KM283-5 and 5D12 antibodies could not suppress DC-MLR. That is, only the KM281-1-10 antibody is an antagonistic antibody that neutralizes the action of CD40 ligands on DC. Moreover, Fig. 11 shows the results of examining the effect of KM302-1 antibodies, which are agonistic antibodies, on MLR using immature DC. Activation of DC promoted interaction with T cells, and caused an increase in thymidine incorporation. These results indicated that KM302-1 is an agonistic antibody that acts on immature DC.

## [Example 11] Effect of CD40 antibody on the binding of CD40L to CD40

[0081] Anti-CD40 antibodies were caused to bind to immobilized CD40 human FC using BIAcore 2000 (Biacore), and then changes in the binding amount of soluble CD40L to CD40 were measured. According to the instruction attached to the system, soluble CD40 human FC was immobilized on a CM chip (CM5, Biacore). Next, 25  $\mu$ g/ml anti-CD40 antibodies were added to bind to CD40. Further, 10  $\mu$ g/ml soluble CD40L was added for binding. A difference between the binding amounts before and after addition of CD40L was measured. When control IgG was added, the binding amount of CD40L was 100 RU. After addition of KM302-1 antibodies, the binding amount of CD40L was 110 RU, and after addition of KM283-5 antibodies the binding amount of CD40L was 18RU. Thus, it was shown that the KM302-1 antibody does not inhibit the binding of CD40L to CD40.

## [Example 12] Promotion of CD95 expression in Ramos cells by anti-CD40 agonistic antibody

[0082] Purified antibodies of the hybridomas obtained in Example 4 were analyzed according to the method of Example 6, and then clones producing agonistic antibodies were selected (number of cells per well:  $5 \times 10^4$ , cell concen-

tration:  $2.5 \times 10^5$ /ml). Figure 12 shows the results. In the figure, the horizontal axis indicates the antibody concentration in culture solutions, and the longitudinal axis indicates average fluorescence intensities, that is, CD95 expression intensities. At a concentration of 0.01  $\mu$ g/ml or more, KM341-1-19 and 2105 antibodies were shown to promote CD95 expression of Ramos cells more effectively than G28-5 antibodies, which are known mouse antibodies. Specifically, KM341-1-19 and 2105 antibodies were shown to be more effective agonistic antibodies. Further, the agonistic activity (to increase CD95 expression of Ramos cells) of KM341-1-19 and 2105 antibodies (0.01  $\mu$ g/ml) was higher than that of G28-5 antibodies (10  $\mu$ g/ml) (Fig. 12). Table 5 summarizes that at each antibody concentration, CD95 expression level is how many times greater or less than that expressed by the addition of G28-5 antibodies.

Table 5

Antibody concentration ( $\mu$ g/ml)	KM341-1-19	2105	F5-77	F2-103
0.01	5.7	3.9		
0.1	7.0	7.1	1.2	1.2
1	5.7	5.1	1.7	1.8
10	4.5	3.3	2.0	1.7

#### [Example 13] Activation of dendritic cell by CD40 agonistic antibody

**[0083]** According to the method of Example 9, the effect of CD40 agonistic antibodies on IL-12 production and IL-10 production by mature DC was examined. IL-10 was measured by the ELISA (Pharmingen) method. Figures 13 and 14 show the results. It was shown that IL-12 secretion was increased by treatment with KM341-1-19 antibodies. In contrast, even when CD40 ligand-expressing recombinant L cells ( $2 \times 10^5$  cells/ml) that had been irradiated with X-rays (5000 rad) were allowed to co-exist, the concentrations of IL-12 and IL-10 in culture solutions were 254 and 51 pg/ml, respectively. They were lower than that when 1  $\mu$ g/ml KM341-1-19 antibodies were added.

**[0084]** As described above, it was shown that KM341-1-19 antibodies act on DC as effective agonistic antibodies. The agonistic activity of KM341-1-19 antibodies (0.1  $\mu$ g/ml) to cause mature DC to secrete IL-12 was higher than that of G28-5 antibodies (100  $\mu$ g/ml). The agonistic activity of KM341-1-19 antibodies (1  $\mu$ g/ml) to cause mature DC to secrete IL-12 was 100 times or more greater than that by G28-5 antibodies (100  $\mu$ g/ml) (Fig. 13). Furthermore, the agonistic activity of KM341-1-19 antibodies (1  $\mu$ g/ml) to cause mature DC to secrete IL-10 was 10 times or more greater than that by G28-5 antibodies (100  $\mu$ g/ml) (Fig. 14). Moreover, since the subclass of KM341-1-19 antibody was IgG2, the antibody has lower binding ability to the Fc receptor than that of IgG1 or IgG3. Its ability to sensitize the killer activity of NK cells and ability to activate the complement system are also weak. Accordingly, there may be a low risk that the function of CD40-expressing cells or the number of the cells themselves decreases due to the antibody. Furthermore, the antibody is not easily cross-linked by an Fc receptor, so that it can be expected that the drug effect is easily controlled without any large fluctuation in in vivo agonistic activity due to cross-linking.

#### [Example 14] Suppression of CD95 expression in Ramos cells by anti-CD40 antagonistic antibody

**[0085]**  $1.0 \times 10^6$  cells/ml Ramos cell suspension was inoculated at 50  $\mu$ l/well to a flat bottom 96-well plate (number of cells per well:  $5 \times 10^4$ ). Purified antibodies diluted with media were added at 100  $\mu$ l/well to a 96-well plate. Human CD40 ligand-expressing recombinant mouse L cells (see Spriggs, M.K. et. al., J. Exp. Med., 176: 1543, 1992; Garrone, P. et. al., J. Exp. Med., 182: 1265, 1995 and the like) were prepared at  $1.0 \times 10^5$  cells/ml. The prepared cells were added at 50  $\mu$ l/well (the number of Ramos cells per well:  $5 \times 10^4$ ; Ramos cell concentration:  $2.5 \times 10^4$  cells/ml; the number of mouse L cells per well:  $5 \times 10^3$ ; mouse cell concentration:  $2.5 \times 10^4$  cells/ml). After overnight culture, the cells were collected, and then analyzed by FACS using R-PE-labeled anti-CD95 antibodies. Figure 15 shows the results. In the figure, the longitudinal axis indicates the average fluorescence intensity, that is, CD95 expression intensity. Whereas the known 5D12 antibodies suppressed the expression slightly, 4D11 antibodies suppressed, even at a concentration of 0.1  $\mu$ g/ml, CD95 expression to the same degree as that of a case of a negative control wherein no CD40L-expressing cells had been added. Moreover, at a concentration of 1  $\mu$ g/ml, 4D11, F4-465 and KM281-1-10 suppressed CD95 expression to the same degree as that of the case of the negative control wherein no CD40L-expressing cells had been added. These results showed that 4D11, F4-465 and KM281-1-10 antibodies are more effective antagonistic antibodies. Table 6 shows relative values of the average fluorescence intensity corresponding to each antibody concentration, when the value of the control case wherein no antagonistic antibodies were added is determined as 100.

Table 6

Antibody concentration ( $\mu\text{g/ml}$ )	5D12	4D11	F4-465	KM281-1-1 0
0.1	77.6	11.8	49.6	60.1
1	72.3	0.01	2.5	7.3
10	69.5	0	1.1	12.6

[Example 15] Anti-tumor effect in Ramos cell transplantation model by anti-CD40 agonistic antibody

**[0086]** Anti-asialo GM1 antibodies were intravenously injected to 5-week-old C.B.17/lcr-scidJc1 mice (CLEA JAPAN). 1 day later,  $5 \times 10^6$  Ramos cells per mouse were intravenously injected as tumor cells. 1 day later, KM302-1 antibodies or anti-human albumin human IgG antibodies as a negative control were intravenously injected. The doses per mouse of KM302-1 antibodies were 1, 10 and 100  $\mu\text{g}$ , and the same of the negative control antibodies was 100  $\mu\text{g}$ . Each of these antibodies was administered once to 5 mice. Figure 16 shows the results. By day 34 after transplantation, all the mice of the negative control-administered group had died, whereas all the 5 mice each of the groups administered with 10  $\mu\text{g}$  and 100  $\mu\text{g}$  of KM302-1 antibodies had been administered to which survived. Thus, the anti-tumor effect of KM302-1 antibodies was confirmed. The KM302-1 antibody is of the IgG4 subclass, so that the Fc receptor-mediated antibody dependent cellular cytotoxicity (ADCC) and activation of the complement system are weak. Despite these characteristics, it was observed that single administration of 10  $\mu\text{g}$  of KM302-1 antibodies prolonged the survival time of tumor-bearing mice.

[Example 16] Ramos cell proliferation suppression by anti-CD40 agonistic antibody

**[0087]** A Ramos cell suspension was prepared at  $1 \times 10^4$  cells/ml in an RPMI1640 medium supplemented with 10%FBS, and then 100  $\mu\text{l}$  of the suspension was apportioned to a 96-well plate. A KM341-1-19 antibody or soluble ligand solution prepared at 20  $\mu\text{g/ml}$  using media was added. Anti-FLAG antibodies (M2) with the same concentration as that of the ligands were allowed to co-exist with the soluble ligands (the concentration in the reaction solution was 10  $\mu\text{g/ml}$ ), thereby enhancing the activity. After 5 days of culturing, 20  $\mu\text{l}$  of MTS reagent (Promega) was added to each well, and then allowed to react for 2 to 3 hours. Differences in absorbance between the cell-free and antibody-free wells and the cell- and antibody-containing wells were measured at a wavelength of 490 nm, thereby measuring viable cell count. Furthermore, the proliferation-suppressing action was compared with that of G28-5 antibodies using a 96-well U-bottomed plate similarly. KM341-1-19 antibodies or G28-5 antibodies prepared at 2  $\mu\text{g/ml}$  using media were added. Figure 17 shows the results. In wells to which KM341-1-19 antibodies had been added, dead cells were observed, the cell number was significantly lower than those of wells to which G28-5 antibodies or the ligands had been added, and the absorbance was also low. These results indicate that the proliferation of tumor cells was suppressed, and cell death was induced.

[Example 17] cDNA cloning of antibody gene

**[0088]** Hybridomas producing KM341-1-19, 2105, 110, 115, KM281-1-10, 4D11, KM643-4-11, F4-465, F2-103 and F5-77 antibodies were cultured, and then the cells were collected by centrifugation. TRIzol (Gibco BRL) was added to the cells, and then Total RNA was extracted according to the attached instructions. Cloning of the variable regions of the antibody cDNA was performed according to the attached instructions using a SMART RACE cDNA amplification Kit (CLONTECH). Using 5  $\mu\text{g}$  of total RNA as a template, 1st Strand cDNA was constructed. To amplify the heavy chains (H chain) of KM341-1-19, 2105, 110, 115, KM281-1-10, 4D11, KM643-4-11, F2-103 and F5-77, Z-Taq (Takara) and UMP and hh6 primers were used, and a cycle of 98°C for 1 second and 68°C for 30 seconds was repeated 30 times. Furthermore, using 1  $\mu\text{l}$  of the reaction solution as a template and NUMP and hh3 primers, a cycle of 98°C for 1 second and 68°C for 30 seconds was repeated 20 times. To amplify a F4-465 heavy chain, UMP and hh2 primers and an Advantage 2 PCR kit (Clontech, cat#1910) were used, and 5 cycles of 94°C for 5 seconds and 72°C for 3 minutes, 5 cycles of 94°C for 5 seconds, 70°C for 0 seconds and 72°C for 3 minutes, and 25 cycles of 94°C for 5 seconds, 68°C for 10 seconds and 72°C for 3 minutes were performed.

hh6 primer: 5'-GGT CCG GGA GAT CAT GAG GGT GTC CTT-3' (SEQ  
ID NO: 3)

hh3 primer: 5'-GTG CAC GCC GCT GGT CAG GGC GCC TG-3' (SEQ ID  
NO: 4)

hh2 primer: 5'-GCT GGA GGG CAC GGT CAC CAC GCT G-3' (SEQ ID  
NO: 5)

[0089] Subsequently, the amplified PCR product was purified using a PCR purification kit (QIAGEN), and then the nucleotide sequence was determined using hh4 as a primer. Alternatively, the product was subcloned to PCR-Script (Stratagene, LaJolla, CA) or PCR-Blunt (Invitrogen, Carlsbad, CA), and then sequencing was performed. Based on the sequence information, antibody heavy-chain-specific primers were synthesized. A 341H primer was synthesized in the case of KM341-1-19, a 2105Hsal primer in the case of 2105, a 110Hsal primer in the case of 110 and 115, a 281Hsal primer in the case of KM281-1-10, a 4D11Sal primer in the case of 4D11, a 643Hsal primer in the case of KM643-4-11, H11-9 5' primer in the case of F4-465, a F2-103H primer in the case of F2-103 and F5-77H primer in the case of F5-77. Using the antibody heavy chain specific primers and hh4, cDNA was amplified from the 1st Strand cDNA, and then the sequence from the opposite direction was determined using the amplified product as a template and the antibody-specific primers.

hh4 primer: 5'-GGTGCCAGGGGGAAGACCGATGG-3' (SEQ ID NO: 6)

341H primer: 5'-atatgtcgacGCTGAATTCTGGCTGACCAGGGCAG-3'  
(SEQ ID NO: 7)

2105Hsal: atatgtcgacTCCCAGGTGTTTCCATTCAGTGATCAG (SEQ ID  
NO: 8)

110Hsal: atatgtcgacTTCCATTCGGTGATCAGCACTGAACAC (SEQ ID  
NO: 9)

281Hsal: atatgtcgacTTTGAGAGTCCTGGACCTCCTGTG (SEQ ID NO:  
10)

4D11Sal: atatgtcgacGAGTCATGGATCTCATGTGCAAG (SEQ ID NO: 11)

643Hsal: atatgtcgacCCAGGGCAGTCACCAGAGCTCCAGAC (SEQ ID  
NO: 12)

H11-9 5': -ACC GTG TCG ACT ACG CGG GAG TGA CT (SEQ ID NO:  
13)

F2-103 H: accgtgtcgacgctgacgactgcaca (SEQ ID NO: 14)

F5-77 H: accgtgtcgacgggtgacgactgaacag (SEQ ID NO: 15)

[0090] The light chains (L chains) of KM341-1-19, 2105, 110, 115, KM281-1-10, 4D11, KM643-4-11, F2-103 and F5-77 were amplified using UMP and hk2 primers and by repeating 30 times a cycle of 98°C for 1 second and 68°C for 30 seconds. The light chain of F4-465 was amplified using UMP and hL2 primers and by repeating 30 times a cycle of 98°C for 1 second and 68°C for 30 seconds. The amplified PCR product was purified using a PCR purification kit, and then the nucleotide sequence was determined using hk6 or hL2 primers. Based on the sequences, light chain specific primers were synthesized. A 341K primer was synthesized in the case of KM341-1-19, 2053KBgl primer in the case of 2105, 110KBgl primer in the case of 110 and 115, 281KBgl primer in the case of KM281-1-10, 4D11KBgl primer in the case of 4D11, 643KBgl primer in the case of KM643-4-11, Lamda 5' primer in the case of F4-465, and F2-103K primer in the case of F-103 and F5-77.

[0091] In the case of 341-1-19, 110, 115, KM643-4-11, KM281-1-10, 4D11 and 2105, cDNA was amplified from the 1st Strand cDNA using the light chain specific primer and hk6 primer. The sequence was then determined from both directions using the amplified product as a template. For F4-465, F2-103 and F5-77, subcloning to PCR-Script (Stratagene, LaJolla, CA) or PCR-Blunt (Invitrogen, Carlsbad, CA) was performed to determine the sequence.

hk2 primer: 5'-GTT GAA GCT CTT TGT GAC GGG CGA GC-3' (SEQ ID  
NO: 16)

hL2 primer: 5'- TCT TCT CCA CGG TGC TCC CTT CAT-3' (SEQ ID NO:  
17)

341K primer: 5'-atatagatctGAACTGCTCAGTTAGGACCCAGAGG-3'  
(SEQ ID NO: 18)

2053KBgl: atatagatctCGCGGGGAAGGAGACTGCTCAGTT (SEQ ID NO:  
19)

110KBgl: atatagatctAGTCAGACCCAGTCAGGACACAGC (SEQ ID NO:  
20)

281KBgl: atatagatctGAGCTGCTCAGTTAGGACCCAGAGGG (SEQ ID  
NO: 21)

4D11KBgl: atatagatctTAAGCAAGTGTAACAACTCAGAGTAC (SEQ ID NO: 22)

643KBgl: atatagatctGAGGAACTGCTCAGTTAGGACCCAGAGG (SEQ ID NO: 23)

Lamda 5': -AACTCCAGATCTGCCTCAGGAAGCAGCATC (SEQ ID NO: 24)

F2-103 K: aactccagatctagggcaagcagtggtaac (SEQ ID NO: 25)

hk6 primer: 5'-TGGCGGGAAGATGAAGACAGATGGTG-3' (SEQ ID NO: 26)

[0092] DNAs of 341-1-19 encoding the full-length H-chain and L-chain variable regions and the amino acid sequences of H-chain and L-chain are respectively shown below.

[0093] The translation initiation point of the H-chain DNA is an ATG codon that begins from the 50th adenine (A) from the 5' end of SEQ ID NO: 27, and the termination codon is TGA beginning from the 1472nd thymine (T). The boundary of the antibody variable region and the constant region is located between the 493rd adenine (A) and the 494th guanine (G) from the 5'end. In the amino acid sequence, the H-chain variable region ranges from the N-terminus to the 148th serine (S) residue of SEQ ID NO: 28, and the constant region is of the 149th alanine (A) and the following residues. It was predicted by a gene sequence prediction software (Signal P ver.2) that the H-chain signal sequence ranges from the N-terminus to the 20th serine (S) of SEQ ID NO: 28. It is thought that the N-terminus of the mature protein is the 21 st glutamine (Q) of SEQ ID NO: 28.

[0094] The translation initiation point of the L-chain DNA is an ATG codon that begins from the 29th A from the 5' end of SEQ ID NO: 29, and the variable region ranges from the 5' end to the 400th adenine (A). In the amino acid sequence, the variable region ranges from the N-terminus to the 124th lysine (K) of SEQ ID NO: 30. Analysis of the N-terminus of the purified L-chain protein revealed that the L-chain signal sequence ranges from the N-terminus to the 20th glycine (G) of SEQ ID NO: 30, and the N-terminus of the mature protein is the 21 st glutamic acid (E) of SEQ ID NO: 30.



341-1-19 H-chain (SEQ ID NO: 27):

5 GTCGACGCTGAATTCTGGCTGACCAGGGCAGCCACCAGAGCTCCAGACAA  
TGTCTGTCTCCTTCCTCATCTTCCTGCCCCGTGCTGGGCCTCCCATGGGGTGT  
CCTGTACACAGGTCCAACCTGCAGCAGTCAGGTCCAGGACTGGTGAAGCCCT  
10 CGCAGACCCTCTCACTCACCTGTGCCATCTCCGGGGACAGTGTCTCTAGCA  
ACAGTGCTACTTGGAACCTGGATCAGGCAGTCCCCATCGAGAGACCTTGAGT  
GGCTGGGAAGGACATACTACAGGTCCAAGTGGTATCGTGATTATGTAGGAT  
15 CTGTGAAAAGTCGAATAATCATCAACCCAGACACATCCAACAACCAGTTCT  
CCCTGCAGCTGAACTCTGTGACTCCCGAGGACACGGCTATATATTACTGTAC  
AAGAGCACAGTGGCTGGGAGGGGATTACCCCTACTACTACAGTATGGACGT  
20

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CTGGGGCCAAGGGACCACGGTCACCGTCTCTTCAGCCTCCACCAAGGGCC  
 5 CATCGGTCTTCCCCCTGGCGCCCTGCTCCAGGAGCACCTCCGAGAGCACAG  
 CGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTG  
 TCGTGGA ACTCAGGCGCTCTGACCAGCGGCGTGACACCTTCCCAGCTGT  
 10 CCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTC  
 CAGCAACTTCGGCACCCAGACCTACACCTGCAACGTA GATCACAAGCCCA  
 GCAACACCAAGGTGGACAAGACAGTTGAGCGCAAATGTTGTGTCGAGTGC  
 15 CCACCGTGCCCAGCACCACTGTGGCAGGACCGTCAGTCTTCCTCTTCCCC  
 CCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTACAGTG  
 CGTGGTGGTGGACGTGAGCCACGAAGACCCCGAGGTCCAGTTCAACTGGT  
 20 ACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCACGGGAGGA  
 GCAGTTCAACAGCACGTTCCGTGTGGTCAGCGTCCTCACCGTTGTGCACCA  
 GGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGGC  
 25 CTCCCAGCCCCCATCGAGAAAACCATCTCCAAAACCAAAGGGCAGCCCCG  
 AGAACCACAGGTGTACACCCTGCCCCCATCCCGGGAGGAGATGACCAAGA  
 ACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTACCCCAGCGACATCG  
 30 CCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACA ACTACAAGACCAC  
 ACCTCCCATGCTGGACTCAGACGGCTCCTTCTTCCTCTACAGCAAGCTCAC  
 35 CGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGA  
 TGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTC  
 CGGGTAAATGAGGATCC  
 40

341-1-19 H-chain amino acid sequence (SEQ ID NO: 28)

45 MSVSFLIFLPVLGLPWGVLSQVQLQQSGPGLVKPSQTLSTCAISGDSVSSNSA  
 TWNWIRQSPSRDLEWLGRYYRSKWYRDYVGSVKSRHINPDTSNNQFSLQL  
 NSVTPEDTAIYYCTRAQWLGGDYPYYYSMDVWGQGTTVTVSSASTKGPSVF  
 50 PLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLY  
 SLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTV ERKCCVECPPCPAPPVAG  
 PSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVQFNWYVDGVEVHNAKT  
 55

KPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKG  
 QPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT  
 PPMLDSDGSFFLYSKLTVDKSRWQQGNVFCSSVMHEALHNHYTQKSLSLSPG  
 K

341-1-19 L-chain (SEQ ID NO: 29):

ACTGCTCAGTTAGGACCCAGAGGGAACCATGGAAGCCCCAGCTCAGCTTC  
 TCTTCCTCCTGCTACTCTGGCTCCCAGATACCACCGGAGAAATTGTGTTGAC  
 ACAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTC  
 CTGCAGGGCCAGTCAGAGTGTTAGCAGCTACTTAGCCTGGTACCAACAGA  
 AACCTGGCCAGGCTCCCAGGCTCCTCATCTATGATGCATCCAACAGGGCCA  
 CTGGCATCCCAGCCAGGTTCAAGTGGCAGTGGGTCTGGGACAGACTTCACT  
 CTCACCATCAGCAGCCTAGAGCCTGAAGATTTTGCAGTTTATTACTGTCAGC  
 AGCGTAGCAACACTTTTCGGCCCTGGGACCAAAGTGGATATCAAACGTACG

341-1-19 L-chain amino acid sequence (SEQ ID NO: 30)

MEAPAQLLFLLLLWLDPDTTGEIVLTQSPATLSLSPGERATLSCRASQSVSSYLA  
 WYQQKPGQAPRLLIYDASNRATGIPARFSGSGSGTDFTLTISLLEPEDFAVYYC  
 QQRSNTFGPGTKVDIKRT

[0095] DNAs of 2105 encoding the H-chain variable region and L-chain variable region and the amino acid sequences of the H-chain and L-chain are shown below.

[0096] The translation initiation point of the H-chain DNA is an ATG codon that begins from the 70th adenine (A) from the 5' end of SEQ ID NO: 31. The boundary of the antibody variable region and the constant region is located between the 495th adenine (A) and the 496th guanine (G) from the 5' end. In the amino acid sequence, the H-chain variable region ranges from the N-terminus to the 142nd serine (S) residue of SEQ ID NO: 32, and the constant region is of the 149th alanine (A) and the following residues. It was predicted by a gene sequence prediction software (Signal P ver.2) that the H-chain signal sequence ranges from the N-terminus to the 19th cysteine (C) of SEQ ID NO: 32. It is thought that the N-terminus of the mature protein is the 20th glutamic acid (E) of SEQ ID NO: 32.

[0097] The translation initiation point of the L-chain DNA is an ATG codon that begins from the 28th A from the 5' end of SEQ ID NO: 33, and the variable region ranges from the 5' end to the 405th adenine (A). In the amino acid sequence, the variable region ranges from the N-terminus to the 126th lysine (K) of SEQ ID NO: 34. It was predicted by gene sequence prediction software (Signal P ver.2) that the L-chain signal sequence ranges from the N-terminus to the 20th glycine (G) of SEQ ID NO: 34. It is thought that the N-terminus of the mature protein is the 21st glutamic acid (E) of SEQ ID NO: 34.

## 2105 H-chain (SEQ ID NO: 31)

CTGAACACAGACCCGTCGACTCCCAGGTGTTTCCATTCAGTGATCAGCACT  
GAACACAGAGGACTCACCATGGAGTTGGGACTGAGCTGGATTTTCCTTTTG  
GCTATTTTAAAAGGTGTCCAGTGTGAAGTGCAGCTGGTGGAGTCTGGGGG  
AGGCTTGGTACAGCCTGGCAGGTCCCTGAGACTCTCCTGTGCAGCCTCTGG  
ATTCACCTTTGATGATTATGCCATGCACTGGGTCCGGCAAGCTCCAGGGAA  
GGGCCTGGAGTGGGTCTCAGGTATTAGTTGGAATAGTGGTAGCTTGGTGCA  
TGCGGACTCTGTGAAGGGCCGATTACCATCTCCAGAGACAACGCCAAGA  
ACTCCCTGTATCTGCAAATGAACAGTCTGAGAGCTGAGGACACGGCCTTGT  
ATTACTGTGCAAGAGATAGGCTATTTTCGGGGAGTTAGGTACTACGGTATGGA  
CGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAGCTAGCACCAAGG

## 2105 H-chain amino acid sequence (SEQ ID NO: 32)

MELGLSWIFLLAILKGVQCEVQLVESGGGLVQPGRSLRLSCAASGFTFDDYA  
MHWVRQAPGKGLEWVSGISWNSGSLVHADSVKGRFTISRDNAKNSLYLQMN

SLRAEDTALYYCARDRLFGRVRYYGMDVWGQGTTVTVSSASTK

## 2105 L-chain (SEQ ID NO: 33)

CTGCTCAGTTAGGACCCAGAGGGAACCATGGAAGCCCCAGCTCAGCTTCT  
CTTCCTCCTGCTACTCTGGCTCCCAGATACCACCGGAGAAATTGTGTTGAC  
ACAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTC  
CTGCAGGGCCAGTCAGAGTGTTAGCAGCTACTTAGCCTGGTACCAACAGA  
AACCTGGCCAGGCTCCCAGGCTCCTCATCTATGATGCATCCAACAGGGCCA  
CTGGCATCCCAGCCAGGTTCACTGGCAGTGGGTCTGGGACAGACTTCACT  
CTCACCATCAGCAGCCTAGAGCCTGAAGATTTTGCAGTTTATTACTGTCAGC  
AGCGTAGCCACTGGCTCACTTTCGGCGGGGGGACCAAGGTGGAGATCAAA  
CGTACGGTG

## 2105 L-chain amino acid sequence (SEQ ID NO: 34)

MEAPAQLLFLLLLWLPD TTGEIVLTQSPATLSLSPGERATLSCRASQSVSSYLA  
 WYQQKPGQAPRL LIYDASN RATGIPARFSGSGSGTDFTLT ISSLEPEDFAVYYC  
 QQRSHWLTFGGG GTKVEIKRTV

[0098] DNAs of 110 encoding the H-chain variable region and L-chain variable region and the amino acid sequences of the H-chain and L-chain are respectively shown below.

[0099] The translation initiation point of the H-chain DNA is an ATG codon that begins from the 60th adenine (A) from the 5' end of SEQ ID NO: 35. The boundary of the antibody variable region and the constant region is located between the 479th adenine (A) and the 480th guanine (G) from the 5' end. In the amino acid sequence, the H-chain variable region ranges from the N-terminus of SEQ ID NO: 36 to the 140th serine (S) residue, and the constant region is of the 141st alanine (A) and the following residues. It was predicted by gene sequence prediction software (Signal P ver.2) that the H-chain signal sequence ranges from the N-terminus to the 19th cysteine (C) of SEQ ID NO: 36. It is thought that the N-terminus of the mature protein is the 20th glutamine (Q) of SEQ ID NO: 36.

[0100] The translation initiation point of the L-chain DNA is an ATG codon that begins from the 35th A from the 5' end of SEQ ID NO: 37, and the variable region ranges from the 5' end to the 421st adenine (A). In the amino acid sequence, the variable region ranges from the N-terminus to the 129th lysine (K) of SEQ ID NO: 38. It was predicted by gene sequence prediction software (Signal P ver.2) that the L-chain signal sequence ranges from the N-terminus to the 22nd cysteine (C) of SEQ ID NO: 38. It is thought that the N-terminus of the mature protein is the 23rd valine (V) of SEQ ID NO: 38.

## 110 H-chain (SEQ ID NO: 35)

CTGAACACAGACCCGTCGACTTCCATTCGGTGATCAGCACTGAACACAGA  
 GGACTCACCATGGAGTTTGGGCTGAGCTGGGTTTTCTCGTTGCTCTTTTA  
 AGAGGTGTCCAGTGT CAGGTGCAGCTGGTGGAGTCTGGGGGAGGCGTGGT  
 CCAGCCTGGGAGGTCCCTGAGACTCTCCTGTGCAGCGTCTGGATTACCTT  
 CAGTAGCTATGGCATGCACTGGGTCCGCCAGGCTCCAGGCAAGGGGCTGG  
 AGTGGGTGGCAGTTATATGGTATGATGGAAGTATTAAATACTATGCAGACTC  
 CGTGAAGGGCCGATTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTA  
 TCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCTGTGTATTACTGTGC  
 GAGAGAGGGGCTACAATATTTTGACTGGTTATTTTGGCTACTGGGGGCCAGGG  
 AACCTGGTCACCGTCTCCTCAGCTAGCACCAAGGG

## 110 H-chain amino acid sequence (SEQ ID NO: 36)

MEFGLSWVFLVALLRGVQCQVQLVESGGGVVQPG RSLRLSCAASGFTFSSYG  
 MHWVRQAPGKGLEWVAVIWDGSIKYYADSVKGRFTISRDN SKNTLYLQMN  
 SLRAEDTAVYYCAREGYNILTGYFGYWGQGLVTVSSASTK

## 110 L-chain (SEQ ID NO: 37)

5 TCACAGATCTAGTCAGACCCAGTCAGGACACAGCATGGACATGAGGGTCC  
 10 CCGCTCAGCTCCTGGGGCTCCTGCTGCTCTGGCTCCCAGGTGCCAGATGTG  
 TCATCTGGATGACCCAGTCTCCATCCTTACTCTCTGCATCTACAGGAGACAG  
 AGTCACCATCAGTTGTCGGATGAGTCAGGGCATTAGCAGTGATTTAGCCTG  
 15 GTATCAGCAAAAACAGGGAAAGCCCCTGAGCTCCTGATCTCTGCTGCATC  
 CACTTTGCAAAGTGGGGTCCCATCAAGGTTCAAGTGGCAGTGGATCTGGGA  
 CAGATTTCACTCTCACCATCAGCTGCCTGCAGTCTGAAGATTTTGCAACTTA  
 20 TTACTGTCAACAGTATTATAGTTTTCCGTGGACGTTTCGGCCAAGGGACCAA  
 GGTGGAAATCAAACGTACG

## 110 L-chain amino acid sequence (SEQ ID NO: 38)

30 MDMRVPAQLLGLLLLWLPGARCVIWMQTQSPSLLSASTGDRVITISCRMSQGISS  
 DLAWYQQKPGKAPELLISAASLTQSGVPSRFSGSGSGTDFTLTISCLQSEDFAT  
 35 YYCQQYYSPWTFGQGTKVEIKRT

**[0101]** DNAs of 115 encoding the H-chain variable region and L-chain variable region and the amino acid sequences of the H-chain and L-chain are respectively shown below.

**[0102]** The translation initiation point of the H-chain DNA is an ATG codon that begins from the 60th adenine (A) from the 5' end of SEQ ID NO: 39. The boundary of the antibody variable region and the constant region is located between the 479th adenine (A) and the 480th guanine (G) from the 5' end. In the amino acid sequence, the H-chain variable region ranges from the N-terminus of SEQ ID NO: 40 to the 140th serine (S) residue, and the constant region is of the 141st alanine (A) and the following residues. It was predicted by gene sequence prediction software (Signal P ver.2) that the H-chain signal sequence ranges from the N-terminus to the 19th cysteine (C) of SEQ ID NO: 40. It is thought that the N-terminus of the mature protein is the 20th glutamine (Q) of SEQ ID NO: 40.

**[0103]** The translation initiation point of the L-chain DNA is an ATG codon that begins from the 35th A from the 5' end of SEQ ID NO: 41, and the variable region ranges from the 5' end to the 421st adenine (A). In the amino acid sequence, the variable region ranges from the N-terminus to the 129th lysine (K) of SEQ ID NO: 42. It was predicted by gene sequence prediction software (Signal P ver.2) that the L-chain signal sequence ranges from the N-terminus to the 22nd cysteine (C) of SEQ ID NO: 42. It is thought that the N-terminus of the mature protein is the 23rd valine (V) of SEQ ID NO: 42.

## 115 H-chain (SEQ ID NO: 39)

5 CTGAACACAGACCCGTCGACTTCCATTCGGTGATCAGCACTGAACACAGA  
GGACTCACCATGGAGTTTGGGCTGAGCTGGGTTTTCTCTCGTTGCTCTTTTA  
AGAGGTGTCCAGTGTGAGGTGCAGCTGGTGAGTCTGGGGGAGGCGTGGT  
10 CCAGCCTGGGAGGTCCCTGAGACTCTCCTGTGCAGCGTCTGGATTACCTT  
CAGTAGCTATGGCATGCACTGGGTCCGCCAGGCTCCAGGCAAGGGGCTGG  
AGTGGGTGGCAGTTATATGGAATGATGGAAGTATTAAATACTATGCAGACTC  
15 CGTGAAGGGCCGATTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTA  
TCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCTGTGTATTACTGTGC  
GAGAGAGGGCTACAATATTTTGACTGGTTATTTTGGCTACTGGGGCCAGGG  
20 AACCTGGTCACCGTCTCCTCAGCTAGCACCAAGG

## 115 H-chain amino acid sequence (SEQ ID NO: 40)

25 MEFGLSWVFLVALLRGVQCQVQLVESGGGVVQPGRSLRLSCAASGFTFSSYG  
MHWVRQAPGKGLEWVAVIWNDGSIKYYADSVKGRFTISRDN SKNTLYLQMN  
30 SLRAEDTAVYYCAREGYNILTGYFGYWGQGLTVTVSSASTK

## 115 L-chain (SEQ ID NO: 41)

35 TCACAGATCTAGTCAGACCCAGTCAGGACACAGCATGGACATGAGGGTCC  
CCGCTCAGCTCCTGGGGCTCCTGCTGCTCTGGCTCCCAGGTGCCAGATGTG  
40 TCATCTGGATGACCCAGTCTCCATCCTTACTCTCTGCATCTACAGGAGACAG  
AGTCACCATCAGTTGTGCGATGAGTCAGGGCATTAGCAGTGATTTAGCCTG  
45 GTATCAGCAAAAACCAGGGAAAGCCCCTGAGCTCCTGATCTCTGCTGCATC  
CACTTTGCAAAGTGGGGTCCCATCAAGGTTCAGTGGCAGTGGATCTGGGA  
CAGATTTCACTCTCACCATCAGCTGCCTGCAGTCTGAAGATTTTGCAACTTA  
50 TTA CTGTCAACAGTATTATAGTTTTCCGTGGACGTTCCGGCCAAGGGACCAA  
GGTGGAATCAAACGTACG  
55

**115 L-chain amino acid sequence (SEQ ID NO: 42)**

MDMRVPAQLLGLLLLWLPGARCVIWM TQSPSLLSASTGDRVTISCRMSQGISS  
 DLAWYQQKPGKAPELLISAASTLQSGVPSRFSGSGSGTDFTLTISCLQSEDFAT  
 YYCQQYYSPWTFGQGTKVEIKRT

**[0104]** DNAs of 281-1-10 encoding the H-chain variable region and L-chain variable region and the amino acid sequences of the H-chain and L-chain are respectively shown below.

**[0105]** The translation initiation point of the H-chain DNA is an ATG codon that begins from the 52nd adenine (A) from the 5' end of SEQ ID NO: 43. The boundary of the antibody variable region and the constant region is located between the 468th adenine (A) and the 469th guanine (G) from the 5' end. In the amino acid sequence, the H-chain variable region ranges from the N-terminus of SEQ ID NO: 44 to the 139th serine (S) residue, and the constant region is of the 140th alanine (A) and the following residues. It was predicted by gene sequence prediction software (Signal P ver.2) that the H-chain signal sequence ranges from the N-terminus to the 19th serine (S) of SEQ ID NO: 44. It is thought that the N-terminus of the mature protein is the 20th glutamine (Q) of SEQ ID NO: 44.

**[0106]** The translation initiation point of the L-chain DNA is an ATG codon that begins from the 41 st A from the 5' end of SEQ ID NO: 45, and the variable region ranges from the 5' end to the 424th adenine (A). In the amino acid sequence, the variable region ranges from the N-terminus to the 128th lysine (K) of SEQ ID NO: 46. It was predicted by gene sequence prediction software (Signal P ver.2) that the L-chain signal sequence ranges from the N-terminus to the 20th glycine (G) of SEQ ID NO: 46. It is thought that the N-terminus of the mature protein is the 21 st glutamic acid (E) of SEQ ID NO: 46.

**281-1-10 H-chain (SEQ ID NO: 43)**

CTGAACACAGACCCGTCGACTTTGAGAGTCCTGGACCTCCTGTGCAAGAA  
 CATGAAACATCTGTGGTTCTTCCTTCTCCTGGTGGCAGCTCCCAGATGGGT  
 CCTGTCCCAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTT  
 CGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTGGCTCCATCAGTGGTT  
 ACTACTGGAGCTGGATCCGGCAGCCCCCAGGGAAGGGACTGGAGTGGATT  
 GGGTATATCTATTACAGTGGGAGCACCAACTACAATCCCTCCCTCAAGAGTC  
 GAGTCACCATATCAGTAGACACGTCCAAGAACCAGTTCTCCCTGAAGCTGA  
 ATTCTGTGACCGCTGCGGACACGGCCGTGTATTACTGTGCGAGAGCCCCCT  
 TGCACGGTGACTACAAATGGTTCCACCCCTGGGGCCAGGGAACCCTGGTC  
 ACCGTCTCCTCAGCTAGCACCAAGG

**281-1-10 H-chain amino acid sequence (SEQ ID NO: 44)**

MKHLWFFLLLVAAPRWVLSQVQLQESGPGLVKPSSETLSLTCTVSGGSISGY  
 WSWIRQPPGKGLEWIGYIYYSGSTNYPNPSLKSRVTISVDTSKNQFSLKLN  
 SVTAADTAVYYCARAPLHGDKWFHPWGQGLVTVSSASTK



## 281-1-10 L-chain (SEQ ID NO: 45)

5 TCACAGATCTGAGCTGCTCAGTTAGGACCCAGAGGGAACCATGGAAACCC  
 CAGCGCAGCTTCTCTTCCTCCTGCTACTCTGGCTCCCAGATACCAACCGGAG  
 AAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAA  
 10 GAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGCAGCTACTTAG  
 CCTGGTACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTG  
 CATCCAGCAGGGCCACTGGCATCCCAGACAGGTTCAGTGGCAGTGGGTCT

20 GGGACAGACTTCACTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCA  
 GTGTATTACTGTCAGCAGTATGGTAGCTCACCGATCACCTTCGGCCAAGGG  
 ACACGACTGGAGATCAAACGTACG

## 281-1-10 L-chain amino acid sequence (SEQ ID NO: 46)

25 METPAQLLFLLLLWLPD TTGEIVLTQSPG TLSLSPGERATLSCRASQSVSSSYL  
 30 AWYQQKPGQAPRL LIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYY  
 CQQYGSSPITFGQGTRLEIKRT

35 **[0107]** DNAs of 4D11 encoding the H-chain variable region and L-chain variable region and the amino acid sequences of the H-chain and L-chain are respectively shown below.

40 **[0108]** The translation initiation point of the H-chain DNA is an ATG codon that begins from the 16th adenine (A) from the 5' end of SEQ ID NO: 47. The boundary of the antibody variable region and the constant region is located between the 456th adenine (A) and the 457th guanine (G) from the 5' end. In the amino acid sequence, the H-chain variable region ranges from the N-terminus of SEQ ID NO: 48 to the 147th serine (S) residue, and the constant region is of the 148th alanine (A) and the following residues. It was predicted by gene sequence prediction software (Signal P ver.2) that the H-chain signal sequence ranges from the N-terminus to the 26th serine (S) of SEQ ID NO: 48. It is thought that the N-terminus of the mature protein is the 27th glutamine (Q) of SEQ ID NO: 48.

45 **[0109]** The translation initiation point of the L-chain DNA is an ATG codon that begins from the 59th A from the 5' end of SEQ ID NO: 49, and the variable region ranges from the 5' end to the 442nd adenine (A). In the amino acid sequence, the variable region ranges from the N-terminus to the 128th lysine (K) of SEQ ID NO: 50. It was predicted by gene sequence prediction software (Signal P ver.2) that the L-chain signal sequence ranges from the N-terminus to the 22nd cysteine (C) of SEQ ID NO: 50. It is thought that the N-terminus of the mature protein is the 21st alanine (A) of SEQ ID NO: 50.

## 4D11 H-chain (SEQ ID NO: 47)

5 ATATGTGCGACGAGTCATGGATCTCATGTGCAAGAAAATGAAGCACCTGTGG  
TTCTTCCTCCTGCTGGTGGCGGCTCCCAGATGGGTCCTGTCCCAGCTGCAG  
CTGCAGGAGTCGGGCCCCAGGACTACTGAAGCCTTCGGAGACCCTGTCCCT  
10 CACCTGCACTGTCTCTGGCGGCTCCATCAGCAGTCCTGGTTACTACGGGGG  
CTGGATCCGCCAGCCCCCAGGGAAGGGGCTGGAGTGGATTGGGAGTATCTA  
TAAAAGTGGGAGCACCTACCACAACCCGTCCCTCAAGAGTCGAGTCACCA  
15 TATCCGTAGACACGTCCAAGAACCAGTTCTCCCTGAAGCTGAGCTCTGTGA  
CCGCCGCAGACACGGCTGTGTATTACTGTACGAGACCTGTAGTACGATATT  
TGGGTGGTTCGACCCCTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAG  
20 CTAGC

## 4D11 H-chain amino acid sequence (SEQ ID NO: 48)

25 MDLMCKKMKHLWFFLLLVAAPRWVLSQLQLQESGPGLLPSETLSLTCTVSG  
GSISSPGYYGGWIRQPPGKGLEWIGSIYKSGSTYHNPSLKSRVTISVDTSKNQF  
30 SLKLSSVTAADTAVYYCTRPPVVRVYFGWFDPWGQGLTVTVSSAS

## 4D11 L-chain (SEQ ID NO: 49)

35 AGATCTTAAGCAAGTGTAACAACTCAGAGTACGCGGGGAGACCCACTCAG  
GACACAGCATGGACATGAGGGTCCCCGCTCAGCTCCTGGGGCTTCTGCTGC  
40 TCTGGCTCCCAGGTGCCAGATGTGCCATCCAGTTGACCCAGTCTCCATCCT  
CCCTGTCTGCATCTGTAGGAGACAGAGTCACCATCACTTGCCGGGCAAGTC  
AGGGCATTAGCAGTGCTTTAGCCTGGTATCAGCAGAAACCAGGGAAAGCT  
45 CCTAAGCTCCTGATCTATGATGCCTCCAATTTGGAAAGTGGGGTCCCATCAA  
GGTTCAGCGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGCC  
50 TGCAGCCTGAAGATTTTGCAACTTATTACTGTCAACAGTTTAATAGTTACCC  
GACGTTTCGGCCAAGGGACCAAGGTGGAAATCAAACGTACG

**4D11 L-chain amino acid sequence (SEQ ID NO: 50)**

MDMRVPAQLLGLLLLWLPGARCAIQLTQSPSSLSASVGDRVTITCRASQGISS  
 ALAWYQQKPGKAPKLLIYDASNLESGVPSRFSGSGSGTDFTLTISSLQPEDFAT  
 YYCQQFNSYPTFGQGTKVEIKRT

[0110] DNAs of KM643-4-11 encoding the H-chain variable region and L-chain variable region and the amino acid sequences of the H-chain and L-chain are respectively shown below.

[0111] The translation initiation point of the H-chain DNA is an ATG codon that begins from the 1 st adenine (A) from the 5' end of SEQ ID NO: 51. The boundary of the antibody variable region and the constant region is located between the 447th adenine (A) and the 448th guanine (G) from the 5' end. In the amino acid sequence, the H-chain variable region ranges from the N-terminus of SEQ ID NO: 52 to the 149th serine (S) residue, and the constant region is of the 150th alanine (A) and the following residues. It was predicted by gene sequence prediction software (Signal P ver.2) that the H-chain signal sequence ranges from the N-terminus to the 20th serine (S) of SEQ ID NO: 52. It is thought that the N-terminus of the mature protein is the 21 st glutamine (Q) of SEQ ID NO: 52.

[0112] The translation initiation point of the L-chain DNA is an ATG codon that begins from the 38th A from the 5' end of SEQ ID NO: 53, and the variable region ranges from the 5' end to the 409th adenine (A). In the amino acid sequence, the variable region ranges from the N-terminus to the 124th lysine (K) of SEQ ID NO: 54. It was predicted by gene sequence prediction software (Signal P ver.2) that the L-chain signal sequence ranges from the N-terminus to the 20th glycine (G) of SEQ ID NO: 54. It is thought that the N-terminus of the mature protein is the 21st Glutamic acid (E) of SEQ ID NO: 54.

**KM643-4-11 H-chain (SEQ ID NO: 51)**

ATGTCTGTCTCCTTCCTCATCTTCCTGCCCCGTGCTGGGCCTCCCATGGGGTG  
 TCCTGTCACAGGTACAGCTGCAGCAGTCAGGTCCAGGACTGGTGAAGCCC  
 TCGCAGACCCTCTCATTACCTGTGCCATCTCCGGGGACAGTGTCTCTAGC  
 AACAGTGCTGCTTGGAACTGGATCAGGCAGTCCCCATCGAGAGGCCTTGA  
 GTGGCTGGGAAGGACATACTACAGGTCCAAGTGGTATAAAGATTATGCAGT  
 ATCTGTGAAAAGTCGAATAACCATCAACCCAGACACATCCAAGAACCAGTT  
 CTCCCTGCAGCTGAACTCTGTGACCCCCGAGGACACGGCTGTGTATTACTG  
 TGCAAGAGGGTATTACTATGGTTCGGGGAGCTATCCCTACTACTACCAAATG  
 GACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAGCTAGC

**KM643-4-11 H-chain amino acid sequence (SEQ ID NO: 52)**

MSVSFLIFLPVLGLPWGVLSQVQLQQSGPGLVKPSQTLSTCAISGDSVSSNSA  
 AWNWIRQSPSRGLEWLGRYYRSKWKDYAVSVKSRITINPDTSKNQFSLQL  
 NSVTPEDTAVYYCARGYYYGSGSYPIYYQMDVWGQGTTVTVSSAS

**KM643-4-11 L-chain (SEQ ID NO: 53)**

5 AATTGAGGAACTGCTCAGTTAGGACCCAGAGGGAACCATGGAAGCCCCAG  
 CTCAGCTTCTCTTCCTCCTGCTACTCTGGCTCCCAGATACCACCGGAGAAAT  
 TGTGTTGACACAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGGGAAAGTGC  
 10 CACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGCTACTTAGCCTGGTA  
 CCAACAGAAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGATGCATCCAA  
 CAGGGCCACTGGCATCCCAGCCAGGTTCAAGTGGCAGTGGGTCTGGGACAG  
 15 ACTTCACTCTCACCATCAGCAGCCTAGAGCCTGAAGATTTTGCAGTTTATTA  
 CTGTCAGCAGCGTAGCAACACTTTCGGCGGAGGGACCAAGGTGGAGATCA  
 AACGAAC

**KM643-4-11 L-chain amino acid sequence (SEQ ID NO: 54)**

25 MEAPAQLLFLLLLWLPD TTGEIVLTQSPATLSLSPGESATLSCRASQSVSSYLA  
 WYQQKPGQAPRLLIYDASN RATGIPARFSGSGSGTDFTLTIS SLEPEDFAVYYC

30 QQRSNTFGGGTKVEIKR

35 **[0113]** DNAs of F4-465 encoding the H-chain variable region and L-chain variable region and the amino acid sequences of the H-chain and L-chain are respectively shown below.

40 **[0114]** The translation initiation point of the H-chain DNA is an ATG codon that begins from the 47th adenine (A) from the 5' end of SEQ ID NO: 55. The boundary of the antibody variable region and the constant region is located between the 484th adenine (A) and the 445th guanine (G) from the 5' end. In the amino acid sequence, the H-chain variable region ranges from the N-terminus of SEQ ID NO: 56 to the 146th serine (S) residue, and the constant region is of the 147th alanine (A) and the following residues. It was predicted by gene sequence prediction software (Signal P ver.2) that the H-chain signal sequence ranges from the N-terminus to the 19th serine (S) of SEQ ID NO: 56. It is thought that the N-terminus of the mature protein is the 20th glutamine (Q) of SEQ ID NO: 56.

45 **[0115]** The translation initiation point of the L-chain DNA is an ATG codon that begins from the 81 st A from the 5' end of SEQ ID NO: 57, and the variable region ranges from the 5' end to the 440th (C). In the amino acid sequence, the variable region ranges from the N-terminus to the 120th Threonine (T) of SEQ ID NO: 58. It was predicted by gene sequence prediction software (Signal P ver.2) that the L-chain signal sequence ranges from the N-terminus to the 19th alanine (G) of SEQ ID NO: 58. It is thought that the N-terminus of the mature protein is the 20th serine (S) of SEQ ID NO: 58.

## F4-465 H-chain (SEQ ID NO: 55)

5 CTGAACACAGACCCGTCGACTACGCGGGAGACCACAGCTCCACACCATGG  
ACTGGACCTGGAGGATCCTATTCTTGGTGGCAGCAGCAACAGGTGCCCCACT  
CCCAGGTGCAGCTGGTGCAATCTGGGTCTGAGTTGAAGAAGCCTGGGGCC  
10 TCAGTGAAGGTCCCCTGCAAGGCTTCTGGATACACCTTCACTAGCTATGCTA  
  
15 TGAATTGGGTGCGACAGGCCCTGGACAAGGGCTTGAGTGGATGGGATGG  
ATCAACACCAACACTGGGAACCAACGTATGCCCAGGGCTTCACAGGACG  
GTTTGTCTTCTCCTTGGACACCTCTGTCAGCACGGCATATCTGCAGATCAGC  
20 AGCCTAAAGGCTGAGGACACTGCCGTGTATTACTGTGCGAGAGAGGTA  
CCAGTTGCTATGAGGGTAACTCACTACTACTACGGTATGGACGTCTGGGGC  
CAAGGGACCACGGTCACCGTCTCCTCAGCTAGCACCAA

## F4-465 H-chain amino acid sequence (SEQ ID NO: 56)

30 MDWTWRILFLVAAATGAHSQVQLVQSGSELKKPGASVKVPCKASGYTFTSYA  
MNWVRQAPGQGLEWMGWINTNTGNPTYAQGFTGRFVFSLDTSVSTAYLQIS  
SLKAEDTAVYYCAREVVPVAMRVTHYYYGMDVWGQGTTVTVSSAST

## F4-465 L-chain (SEQ ID NO: 57)

CTGGGTACGGTAACCGTCAGATCGCCTGGAGACGCCATCACAGATCTGCCT  
 CAGGAAGCAGCATCGGAGGTGCCTCAGCCATGGCATGGATCCCTCTCTTCC  
 TCGGCGTCCTTGTTTACTGCACAGGATCCGTGGCCTCCTATGAGCTGACTC  
 AGCCACCCTCAGTGTCCGTGGCCCCAGGACAGACAGCCAGCATCACCTGT  
 TCTGGAGATAAATTGGGGGATAATTTTACTTGCTGGTATCAGCAGAAGCCA  
 GGCCAGTCCCCTGTGCTGGTCATCTTTCAGGATTGGAAGCGGCGCCAGGG  
 ATCCCTGCGCGATTCTCTGGCTCCAAGTCTGGGAACACAGCCACTCTGACC  
 ATCAGCGGGACCCAGGCTATGGATGAGGCTGACTATTACTGTCAGGCGTGG  
 GACATCAGCACTGTGGTATTCGGCGGAGGGACCAAGCTGACCGTCCTAGGT  
 CAGCCCAAGGCTGCCCCCTCGGTCACTCTGTTCCCGCCCTCCTCTGAGGAG  
 CTTCAAGCCAACAAGGCCACACTGGTGTGTCTCATAAGTGACTTCTACCCG  
 GGAGCCGTGACAGTGGCCTGGAAGGCAGATAGCAGCCCCGTCAAGGCGGG  
 AGTGGAGACCACCACACCCTCCAAACAAAGCAACAACAAGTACGCGGCC  
 AGCAGCTACCTGAGCCTGACGCCTGAGCAGTGGAAGTCCACAGAAGCTA  
 CAGCTGCCAGGTCACGCATGAAGGGAGACCGTGGAGAAGACAGTGGCCC  
 CTACAGAATGTTTCATGAATTCAGATCCGTAAACGGTTACCAACTACCTAGAC

TGGATTCGTGACCAACATA

## F4-465 L-chain amino acid sequence (SEQ ID NO: 58)

MAWIPLFLGVLVYCTGSVASIELTQPPSVSVAPGQTASITCSGDKLGDNFTCW  
 YQQKPGQSPVLVIFQDWKRRPGIPARFSGSKSGNTATLTISGTQAMDEADYYC  
 QAWDISTVVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFYP  
 GAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSC  
 QVTHEGSTVEKTVAPTECS

**[0116]** DNAs of F2-103 encoding the H-chain variable region and L-chain variable region and the amino acid sequences of the H-chain and L-chain are respectively shown below.

**[0117]** The translation initiation point of the H-chain DNA is an ATG codon that begins from the 32nd adenine (A) from the 5' end of SEQ ID NO: 59. The boundary of the antibody variable region and the constant region is located between the 463rd adenine (A) and the 464th Guanine (G) from the 5' end. In the amino acid sequence, the H-chain variable region ranges from the N-terminus of SEQ ID NO: 60 to the 144th Serine (S) residue, and the constant region is of the 145th Alanine (A) and the following residues. It was predicted by gene sequence prediction software (Signal P ver.2) that the H-chain signal sequence ranges from the N-terminus to the 19th Cysteine (C) of SEQ ID NO: 60. It is thought that the N-terminus of the mature protein is the 20th Glutamic acid (E) of SEQ ID NO: 60.

[0118] The translation initiation point of the L-chain DNA is an ATG codon that begins from the 29th A from the 5' end of SEQ ID NO: 61, and the variable region ranges from the 5' end to the 415th adenine (A). In the amino acid sequence, the variable region ranges from the N-terminus to the 129th Lysine (K) of SEQ ID NO: 62. It was predicted by gene sequence prediction software (Signal P ver.2) that the L-chain signal sequence ranges from the N-terminus to the 22nd Cystein (C) of SEQ ID NO: 62. It is thought that the N-terminus of the mature protein is the 23rd Asp (D) of SEQ ID NO: 62.

F2-103 H-chain (SEQ ID NO: 59)

GCTGATCAGGACTGCACACAGAGAACTCACCATGGAGTTTGGGCTGAGCT  
GGGTTTTCTTGTGCTATTTTAAAAGGTGTCCAGTGTGAGGTGCAGCTGG  
TGGAGTCCGGGGGAGGCTTAGTTCAGCCTGGGGGGTCCCTGAGACTCTCC  
TGTGCAGTCTCTGGATTCACCTTCAGTACCTACTGGATGCACTGGGTCCGC  
CAAGCTCCAGGGAAGGGGCTGGTGTGGGTCTCACGTATTAATAGTGATGGG  
AGTAGCACAACCTACGCGGACTCCGTGAAGGGCCGATTCACCATCTCCAGA  
GACAACGCCAAGAACACGCTGTATCTGCAAATGAACAGTCTGAGAGCCGA  
GGACACGGCTGTGTATTACTGTGCAAGAGATAGAGTACTATGGATCGGGGA  
GTTATCCTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGT  
CTCCTCAGCTAGCACCAAGGGGCCCATCGGTCTTCCCCCTGGCACCCCTCCTC  
CAAGAGCACCTCT

F2-103 H-chain amino acid sequence (SEQ ID NO: 60)

MEFGLSWVFLVAILKGVQCEVQLVESGGGLVQPGGSLRLSCAVSGFTFSTYW  
MHWVRQAPGKGLVWVSRINSDGSSTTYADSVKGRFTISRDNKNTLYLQMN  
SLRAEDTAVYYCARDRLVWIGELSYYGMDVWGQGTTVTVSSASTKGPSVFPL  
APSSKSTS

## F2-103 L-chain (SEQ ID NO: 61)

5 GGGGAGTCAGACCCAGTCAGGACACAGCATGGACATGAGGGTCCCCGCTC  
 AGCTCCTGGGGCTCCTGCTGCTCTGGCTCCCAGGTGCCAAATGTGACATCC  
 AGATGACCCAGTCTCCTTCCACCCTGTCTGCATCTGTAGGAGACAGAGTCA  
 10 CCATCACTTGCCGGGGCCAGTCAGAGTATTAGTAACTGGTTGGCCTGGTATC  
 AGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGCTCTATAAGGCATCTGGTT  
 TAGAAAGTGGGGTCCCATCAAGGTTTCAGCGGCAGTGGATCTGGGACAGAA

20 TTCACTCTCACCATCAACAGCCTGCAGCCTGATGATTTTGCAACTTATTACT  
 GCCAACAGTCTAATAGTTATTCGTGGACGTTTCGGCCACGGGACCAAGGTGG  
 AAATCAAACGTACGGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGA  
 25 TGAGCAGTTGAAATCTGGAAGTGCCTCTGTTGTGTGCCTGCTGAATAACTT  
 CTATCCCAGAGAGGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATC  
 GGGTAACTCCCAGGAGAGTGTACAGAGCAGGACAGCAAGGACAGCACCT  
 30 ACAGCCTCAGCAGCACCTGACGCTGAGCAAAGCAGACTACGAGAAACAC  
 AAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGA

## F2-103 L-chain amino acid sequence (SEQ ID NO: 62)

40 MDMRVPAQLLGLLLLWLPGAKCDIQMTQSPSTLSASVGDRVTITCRASQSISN  
 WLAWYQQKPGKAPKLLLYKASGLESQVPSRFSGSGSGTEFTLTINSLQPDDFA  
 TYVCQQSNSYSWTFGHGKVEIKRTVAAPSVFIFPPSDEQLKSGTASVCLLN  
 45 NFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSSTYSLSSTLTLSKADYEKH  
 KVYACEVTHQGL

**[0119]** DNAs of F5-77 encoding the H-chain variable region and L-chain variable region and the amino acid sequences of the H-chain and L-chain are respectively shown below.

**[0120]** The translation initiation point of the H-chain DNA is an ATG codon that begins from the 100th adenine (A) from the 5' end of SEQ ID NO: 63. The boundary of the antibody variable region and the constant region is located between the 528th adenine (A) and the 529th Guanine (G) from the 5' end. In the amino acid sequence, the H-chain variable region ranges from the N-terminus of SEQ ID NO: 64 to the 143rd Serine (S) residue, and the constant region is of the 144th Alanine (A) and the following residues. It was predicted by gene sequence prediction software (Signal P ver.2) that the H-chain signal sequence ranges from the N-terminus to the 19th Cysteine (C) of SEQ ID NO: 64. It is thought that the N-terminus of the mature protein is the 20th Glutamic acid (E) of SEQ ID NO: 64.

**[0121]** The translation initiation point of the L-chain DNA is an ATG codon that begins from the 59th A from the 5'



end of SEQ ID NO: 65, and the variable region ranges from the 5' end to the 445th adenine (A). In the amino acid sequence, the variable region ranges from the N-terminus to the 129th Lysine (K) of SEQ ID NO: 66. It was predicted by gene sequence prediction software (Signal P ver.2) that the L-chain signal sequence ranges from the N-terminus to the 22nd Cystein (C) of SEQ ID NO: 66. It is thought that the N-terminus of the mature protein is the 23rd Asp (D) of SEQ ID NO: 66.

## F5-77 H-chain (SEQ ID NO: 63)

GGTCTATATAAGCAGAGCTGGGTACGTCCTCACATTCAGTGATCAGCACTG  
AACACAGACCCGTCGACGGTGATCAGGACTGAACAGAGAGAACTCACCAT  
GGAGTTTGGGCTGAGCTGGCTTTTTCTTGTGGCTATTTTAAAAGGTGTCCA  
GTGTGAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGG  
GGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGC  
CATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAG  
CTATTAGTGGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCC  
GGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGA  
ACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAGATGGG  
GGGTACTATGGTTCGGGGAGTTATGGGTACTTTGACTACTGGGGCCAGGGA  
ACCCTGGTCACCGTCTCCTCAGCTAGCACCAAGGGCCCATCGGTCTTCCCC  
CTGGCACCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTG  
CCTGGTCAAGGACTACTTCCCC

## F5-77 H-chain amino acid sequence (SEQ ID NO: 64)

MEFGLSWLFLVAILKGVQCEVQLLESGGGLVQPGSLRLSCAASGFTFSSYAM  
SWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDN SKNTLYLQMNSL  
RAEDTAVYYCAKDGGYYGSGSYGYFDYWGGTGLVTVSSASTKGPSVFPLAP

SSKSTSGGTAALGCLVKDYFP

## F5-77 L-chain (SEQ ID NO: 65)

CAAGCAGTGGTAACAACGCAGAGTACGCGGGGGGAGTCAGACCCAGTCA  
 5 GGACACAGCATGGACATGAGGGTCCCCGCTCAGCTCCTGGGGCTCCTGCT  
 GCTCTGGTTCCCAGGTTCCAGATGCGACATCCAGATGACCCAGTCTCCATC  
 10 TTCCGTGTCTGGATCTGTAGGAGACAGAGTCACCATCACTTGTGGGGCGAG  
 TCAGGGTATTAGCAGCTGGTTAGCCTGGTATCAGCAGAAACCAGGGAAAG  
 CCCCTAAGCTCCTGATCTATGCTGGATCCAGTTTGCAAAGTGGGGTCCCATC  
 15 AAGGTTCAAGCGGCAGTGGATTTGGGACAGATTTCACTCTCACCATCAGCAG  
 CCTGCAGCCTGAAGATTTTGCAACTTACTATTGTCAACAGGCTAGCAGTTT  
 CCCTCGGACATTCGGCCAAGGGACCAAGGTGGAGATCAAACGTACGGTGG  
 20 CTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGG  
 AACTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAA  
 AGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGA  
 25 GTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACC  
 CTGACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTACGCCTGCGA  
 30 AGTCACCCATCAGGGCCTGA

## F5-77 L-chain amino acid sequence (SEQ ID NO: 66)

MDMRVPAQLLGLLLLWFPGRCDIQMTQSPSSVSGSVGDRVITICRASQGISS  
 35 WLAWYQQKPGKAPKLLIYAGSSLSQSGVPSRFSGSGFGTDFLTISLQPEDFAT  
 40 YYCQQASSFPRTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNF  
 YPREAKVQWKVDNALQSGNSQESVTEQDSKSTYLSSTLTLSKADYEKHK  
 45 VYACEVTHQGL

[Example 18) Expression of antibody protein in animal cell

[0122] The above obtained DNA fragment containing the variable region of the antibody was incorporated into an  
 50 appropriate vector such as NSKG1 (IDEC Pharmaceuticals, US patent 6001358), thereby preparing an antibody ex-  
 pression vector. As a host cell for expression, for example, CHO-Ras (Katakura Y., et al., Cytotechnology, 31: 103-109,  
 1999) is appropriately used. The vector can be introduced into the host cell by, for example, electroporation. Approx-  
 imately 2 µg of the antibody expression vector was linearized with a restriction enzyme. The gene was introduced into  
 55 4x10<sup>7</sup> CHO-Ras cells under conditions of 350V and 500µF using a Bio-Rad electrophoreter, and then inoculated to a  
 96-well culture plate. A drug corresponding to the selection marker of the expression vector was added, and culturing  
 was continued. When colonies were observed, antibody-expressing lines were selected by the method described in  
 Example 4. Antibodies can be purified from the selected cells according to Example 5.

**[Example 19] Antigen-specific antibody production suppressive action of CD40 antagonistic antibody**

**[0123]** Mice having a genetic background whereby they were homozygotes for mouse endogenous disrupted CD40 and harboring a transgene of a human CD40 gene (Yasui. et al. Int. Immunol. 2002 Vol 14: 319) were sensitized by intraperitoneally injecting 100 µg (in an amount of NP-CGG) of a complex of 4-hydroxy-3-nitrophenylacetyl-chicken γ-globulin conjugates (NP-CGG : distributed by Hitoshi KIKUTANI, Professor, Research Institute for Microbial Diseases, Osaka University) and ARAM (ARAM: Antigen Recognition Activation Motif). 30 or 100 µg of each monoclonal antibody was administered via caudate vein immediately before antigen sensitization. 100 µg of anti-human albumin human IgG4 antibody was administered as a negative control. 7 days after sensitization, blood was collected from the orbital venous plexus, and then the amounts of NP specific IgG1 and IgM antibodies in sera were measured by the ELISA method. The ELISA method was performed by adding NP-bound bovine serum albumin (NP-BSA: 2.5 µg/ml) (50 µl/well) to each well of a 96-well micro plate for ELISA (Maxisorp, Nunc), incubating at 4°C and then absorbing NP-BSA. Next, the supernatant was discarded, a blocking reagent (Super Block, Pierce) was added to each well, and then incubation was performed at room temperature for blocking. Each well was then washed 3 times with a 0.1% Tween20-containing phosphate buffer (PBS-T). Subsequently, each serum diluted with 10% BlockAce-containing PBS-T was added (50 µl/well) to each well, followed by incubation at 37°C for 2 hours for reaction. The microplate was washed 3 times with PBS-T. A solution prepared by diluting 1,000-fold alkaline phosphatase-labeled goat anti-mouse IgG1 or IgM antibody (COSMO BIO, 1070-04 or 1020-04) with 10% BlockAce-containing PBS-T was added (50 µl/well) to each well, followed by 2 hours of incubation at 37°C. Next, the microplate was washed 3 times with PBS-T, a chromogenic substrate solution (50 µl/well, Sigma104, phosphatase substrate) was added to each well, and then absorbance at a wavelength of 405 nm was measured with a microplate reader. Figures 18 and 19 show the results. In the figures, longitudinal axes indicate values obtained by conversion using as a unit the serum diluted 10,000-fold in the case of IgG1 antibody, and the serum diluted 100-fold in the case of IgM antibody. Here, the serum was prepared by injecting NP-CGG twice into C57BL/6 mice, collecting blood from the mice, and pooling the serum. Administration of 100 µg each of F4-465, 4D11 and KM281-1-10 antibodies strongly suppressed NP-specific IgG 1 and IgM antibody production.

**[Example 20] Proliferation suppression of tonsillar B cells by CD40 antagonistic antibody**

**[0124]** Human tonsils were obtained from Children's Hospital (San Diego, California, U.S.A.). Tonsils were cut into small pieces, minced, and then passed through a 70-micrometer nylon mesh cell strainer, thereby preparing a cell suspension. The cell suspension was washed several times with PBS, the cell number was counted, and then the suspension was cryopreserved with 90% human serum (ICN) and 10% DMSO. After thawing, the cells were re-suspended in a standard RPMI medium supplemented with 10% human serum and 2.5 µg/ml amphotericin (fangizon, Gibco/BRL), and then used.

**[0125]**  $1 \times 10^5$  cells were added to a 96-well plate, and then anti-human CD40 antibodies were added at concentrations of 0.01, 0.1, 1.0 and 10 µg/ml. The test was conducted in triplicate at each concentration. 1 µg/ml flag-labeled CD40L (Alexis) and 1 µg/ml CD40L enhancer antibodies (Alexis) were added to each well, followed by 3 days of culturing. Then, 1 µCi [ $^3$ H] thymidine was added to each well. 12 to 15 hours later, the cells were collected, and then the proliferation of tonsillar B cells was measured using a liquid scintillation counter. The count obtained when B cells had proliferated due to the stimulation of CD40L and no antibody had been added was determined as 100, and the count obtained when no CD40L had been added and B cells had not been not stimulated was determined as 0. For example, when the relative count measured was 30, this was expressed in this experiment as that 70% proliferation inhibition had occurred.

**[0126]** 5D12, which is the known antagonistic antibody, did not show more than 50% proliferation inhibition even with the antibody concentration of 100 µg/ml. F4-465 showed approximately 80% proliferation suppression even with the antibody concentration as low as 0.01 µg/ml, and showed approximately 95% proliferation suppression with an antibody concentration of 0.1 to 10 µg/ml (Fig. 20).

**[0127]** All publications, patents and patent applications cited herein are incorporated herein by reference in their entirety.

**Industrial Applicability**

**[0128]** The present invention provides an antibody against CD40. The antibody of the present invention includes both an antibody that acts agonistically on CD40 and an antibody that acts antagonistically on CD40. Thus, these antibodies are useful as, for example, an immunopotentiating agent and immunosuppressive agent, respectively.

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EP 1 391 464 A1

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5 Tyr Tyr Ser Phe Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu Ile  
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10 Lys Arg Thr  
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15 <210> 39  
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<212> DNA  
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45 Met Glu Phe Gly Leu Ser Trp Val Phe Leu Val Ala Leu Leu Arg Gly  
1 5 10 15

50 Val Gln Cys Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln  
20 25 30

55 Pro Gly Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe  
35 40 45

60 Ser Ser Tyr Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu

50

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5 Glu Trp Val Ala Val Ile Trp Asn Asp Gly Ser Ile Lys Tyr Tyr Ala  
65 70 75 80

10 Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn  
85 90 95

15 Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val  
100 105 110

20 Tyr Tyr Cys Ala Arg Glu Gly Tyr Asn Ile Leu Thr Gly Tyr Phe Gly  
115 120 125

25 Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys  
130 135 140

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&lt;210&gt; 41

&lt;211&gt; 427

30 &lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 41

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45 acgtacg 427

50 &lt;210&gt; 42

&lt;211&gt; 131

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

55 &lt;400&gt; 42

Met Asp Met Arg Val Pro Ala Gln Leu Leu Gly Leu Leu Leu Trp

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<210> 44

<211> 143

<212> PRT

<213> Homo sapiens

<400> 44

Met Lys His Leu Trp Phe Phe Leu Leu Leu Val Ala Ala Pro Arg Trp  
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Val Leu Ser Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys  
20 25 30

Pro Ser Glu Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Gly Ser Ile  
35 40 45

Ser Gly Tyr Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Leu  
50 55 60

Glu Trp Ile Gly Tyr Ile Tyr Tyr Ser Gly Ser Thr Asn Tyr Asn Pro  
65 70 75 80

Ser Leu Lys Ser Arg Val Thr Ile Ser Val Asp Thr Ser Lys Asn Gln  
85 90 95

Phe Ser Leu Lys Leu Asn Ser Val Thr Ala Ala Asp Thr Ala Val Tyr  
100 105 110

Tyr Cys Ala Arg Ala Pro Leu His Gly Asp Tyr Lys Trp Phe His Pro  
115 120 125

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys  
130 135 140

<210> 45

<211> 430

<212> DNA

<213> Homo sapiens

<400> 45

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<210> 46

<211> 130

<212> PRT

<213> Homo sapiens

<400> 46

Met Glu Thr Pro Ala Gln Leu Leu Phe Leu Leu Leu Trp Leu Pro  
 1 5 10 15

Asp Thr Thr Gly Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser  
 20 25 30

Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser  
 35 40 45

Val Ser Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala  
 50 55 60

Pro Arg Leu Leu Ile Tyr Gly Ala Ser Ser Arg Ala Thr Gly Ile Pro  
 65 70 75 80

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile  
 85 90 95

Ser Arg Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Tyr  
 100 105 110

Gly Ser Ser Pro Ile Thr Phe Gly Gln Gly Thr Arg Leu Glu Ile Lys  
 115 120 125

Arg Thr  
 130

&lt;210&gt; 47

&lt;211&gt; 462

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 47

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&lt;210&gt; 48

&lt;211&gt; 149

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 48

Met Asp Leu Met Cys Lys Lys Met Lys His Leu Trp Phe Phe Leu Leu  
 1 5 10 15  
 Leu Val Ala Ala Pro Arg Trp Val Leu Ser Gln Leu Gln Leu Gln Glu  
 20 25 30  
 Ser Gly Pro Gly Leu Leu Lys Pro Ser Glu Thr Leu Ser Leu Thr Cys  
 35 40 45  
 Thr Val Ser Gly Gly Ser Ile Ser Ser Pro Gly Tyr Tyr Gly Gly Trp  
 50 55 60  
 Ile Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp Ile Gly Ser Ile Tyr  
 65 70 75 80  
 Lys Ser Gly Ser Thr Tyr His Asn Pro Ser Leu Lys Ser Arg Val Thr  
 85 90 95

Ile Ser Val Asp Thr Ser Lys Asn Gln Phe Ser Leu Lys Leu Ser Ser  
 100 105 110

Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys Thr Arg Pro Val Val  
 115 120 125

Arg Tyr Phe Gly Trp Phe Asp Pro Trp Gly Gln Gly Thr Leu Val Thr  
 130 135 140

Val Ser Ser Ala Ser  
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<210> 49  
 <211> 448  
 <212> DNA  
 <213> Homo sapiens

<400> 49  
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 agggaccaag gtggaaatca aacgtacg 448

<210> 50  
 <211> 130  
 <212> PRT  
 <213> Homo sapiens

<400> 50  
 Met Asp Met Arg Val Pro Ala Gln Leu Leu Gly Leu Leu Leu Leu Trp  
 1 5 10 15

Leu Pro Gly Ala Arg Cys Ala Ile Gln Leu Thr Gln Ser Pro Ser Ser  
 20 25 30

Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser

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5 Gln Gly Ile Ser Ser Ala Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys  
50 55 60

10 Ala Pro Lys Leu Leu Ile Tyr Asp Ala Ser Asn Leu Glu Ser Gly Val  
65 70 75 80

15 Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr  
85 90 95

20 Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln  
100 105 110

Phe Asn Ser Tyr Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys  
115 120 125

25 Arg Thr  
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30 <210> 51  
<211> 453  
<212> DNA  
35 <213> Homo sapiens

<400> 51  
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cagtccecat cgagaggcct tgagtggctg ggaaggacat actacaggtc caagtggat 240  
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45 cagttctccc tgcagctgaa ctctgtgacc cccgaggaca cggctgtgta ttactgtgca 360  
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50 <210> 52  
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55 <213> Homo sapiens

&lt;400&gt; 52

Met Ser Val Ser Phe Leu Ile Phe Leu Pro Val Leu Gly Leu Pro Trp  
 1 5 10 15

Gly Val Leu Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Gly Leu Val  
 20 25 30

Lys Pro Ser Gln Thr Leu Ser Phe Thr Cys Ala Ile Ser Gly Asp Ser  
 35 40 45

Val Ser Ser Asn Ser Ala Ala Trp Asn Trp Ile Arg Gln Ser Pro Ser  
 50 55 60

Arg Gly Leu Glu Trp Leu Gly Arg Thr Tyr Tyr Arg Ser Lys Trp Tyr  
 65 70 75 80

Lys Asp Tyr Ala Val Ser Val Lys Ser Arg Ile Thr Ile Asn Pro Asp  
 85 90 95

Thr Ser Lys Asn Gln Phe Ser Leu Gln Leu Asn Ser Val Thr Pro Glu  
 100 105 110

Asp Thr Ala Val Tyr Tyr Cys Ala Arg Gly Tyr Tyr Tyr Gly Ser Gly  
 115 120 125

Ser Tyr Pro Tyr Tyr Tyr Gln Met Asp Val Trp Gly Gln Gly Thr Thr  
 130 135 140

Val Thr Val Ser Ser Ala Ser  
 145 150

&lt;210&gt; 53

&lt;211&gt; 414

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 53

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10 <210> 54  
 <211> 125  
 <212> PRT  
 <213> Homo sapiens

15 <400> 54  
 Met Glu Ala Pro Ala Gln Leu Leu Phe Leu Leu Leu Leu Trp Leu Pro  
 1 5 10 15

20 Asp Thr Thr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser  
 20 25 30

25 Leu Ser Pro Gly Glu Ser Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser  
 35 40 45

30 Val Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro  
 50 55 60

35 Arg Leu Leu Ile Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala  
 65 70 75 80

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser  
 85 90 95

40 Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser  
 100 105 110

45 Asn Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys Arg  
 115 120 125

50 <210> 55  
 <211> 495  
 <212> DNA  
 <213> Homo sapiens

55 <400> 55

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 10 aaaggctgag gacactgccg tgtattactg tgcgagagag gtagtaccag ttgctatgag 420  
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<210> 56

<211> 149

<212> PRT

<213> Homo sapiens

<400> 56

25 Met Asp Trp Thr Trp Arg Ile Leu Phe Leu Val Ala Ala Ala Thr Gly  
 1 5 10 15

30 Ala His Ser Gln Val Gln Leu Val Gln Ser Gly Ser Glu Leu Lys Lys  
 20 25 30

35 Pro Gly Ala Ser Val Lys Val Pro Cys Lys Ala Ser Gly Tyr Thr Phe  
 35 40 45

40 Thr Ser Tyr Ala Met Asn Trp Val Arg Gln Ala Pro Gly Gln Gly Leu  
 50 55 60

45 Glu Trp Met Gly Trp Ile Asn Thr Asn Thr Gly Asn Pro Thr Tyr Ala  
 65 70 75 80

50 Gln Gly Phe Thr Gly Arg Phe Val Phe Ser Leu Asp Thr Ser Val Ser  
 85 90 95

55 Thr Ala Tyr Leu Gln Ile Ser Ser Leu Lys Ala Glu Asp Thr Ala Val  
 100 105 110

Tyr Tyr Cys Ala Arg Glu Val Val Pro Val Ala Met Arg Val Thr His  
 115 120 125

55 Tyr Tyr Tyr Gly Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val



130

135

140

Ser Ser Ala Ser Thr  
145

&lt;210&gt; 57

&lt;211&gt; 830

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 57

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&lt;210&gt; 58

&lt;211&gt; 231

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 58

Met Ala Trp Ile Pro Leu Phe Leu Gly Val Leu Val Tyr Cys Thr Gly  
1 5 10 15

Ser Val Ala Ser Tyr Glu Leu Thr Gln Pro Pro Ser Val Ser Val Ala  
20 25 30

Pro Gly Gln Thr Ala Ser Ile Thr Cys Ser Gly Asp Lys Leu Gly Asp  
35 40 45

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Asn Phe Thr Cys Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Val Leu  
50 55 60

Val Ile Phe Gln Asp Trp Lys Arg Arg Pro Gly Ile Pro Ala Arg Phe  
65 70 75 80

Ser Gly Ser Lys Ser Gly Asn Thr Ala Thr Leu Thr Ile Ser Gly Thr  
85 90 95

Gln Ala Met Asp Glu Ala Asp Tyr Tyr Cys Gln Ala Trp Asp Ile Ser  
100 105 110

Thr Val Val Phe Gly Gly Gly Thr Lys Leu Thr Val Leu Gly Gln Pro  
115 120 125

Lys Ala Ala Pro Ser Val Thr Leu Phe Pro Pro Ser Ser Glu Glu Leu  
130 135 140

Gln Ala Asn Lys Ala Thr Leu Val Cys Leu Ile Ser Asp Phe Tyr Pro  
145 150 155 160

Gly Ala Val Thr Val Ala Trp Lys Ala Asp Ser Ser Pro Val Lys Ala  
165 170 175

Gly Val Glu Thr Thr Thr Pro Ser Lys Gln Ser Asn Asn Lys Tyr Ala  
180 185 190

Ala Ser Ser Tyr Leu Ser Leu Thr Pro Glu Gln Trp Lys Ser His Arg  
195 200 205

Ser Tyr Ser Cys Gln Val Thr His Glu Gly Ser Thr Val Glu Lys Thr  
210 215 220

Val Ala Pro Thr Glu Cys Ser  
225 230

<210> 59

<211> 520

<212> DNA

<213> Homo sapiens

&lt;400&gt; 59

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 tatggacgtc tggggccaag ggaccacggt caccgtctcc tcagctagca ccaagggcc 480  
 15 atcggctctc cccctggcac cctcctccaa gaggacctct 520

&lt;210&gt; 60

&lt;211&gt; 163

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 60

Met Glu Phe Gly Leu Ser Trp Val Phe Leu Val Ala Ile Leu Lys Gly  
 1 5 10 15

Val Gln Cys Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln  
 20 25 30

Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Val Ser Gly Phe Thr Phe  
 35 40 45

Ser Thr Tyr Trp Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu  
 50 55 60

Val Trp Val Ser Arg Ile Asn Ser Asp Gly Ser Ser Thr Thr Tyr Ala  
 65 70 75 80

Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn  
 85 90 95

Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val  
 100 105 110

Tyr Tyr Cys Ala Arg Asp Arg Val Leu Trp Ile Gly Glu Leu Ser Tyr  
 115 120 125

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Tyr Gly Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser  
130 135 140

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys  
145 150 155 160

Ser Thr Ser

<210> 61

<211> 698

<212> DNA

<213> Homo sapiens

<400> 61

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<210> 62

<211> 223

<212> PRT

<213> Homo sapiens

<400> 62

Met Asp Met Arg Val Pro Ala Gln Leu Leu Gly Leu Leu Leu Leu Trp  
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Leu Pro Gly Ala Lys Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Thr  
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Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser  
                   35                  40                  45

Gln Ser Ile Ser Asn Trp Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys  
           50                  55                  60

Ala Pro Lys Leu Leu Leu Tyr Lys Ala Ser Gly Leu Glu Ser Gly Val  
   65                  70                  75                  80

Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr  
                   85                  90                  95

Ile Asn Ser Leu Gln Pro Asp Asp Phe Ala Thr Tyr Tyr Cys Gln Gln  
                   100                  105                  110

Ser Asn Ser Tyr Ser Trp Thr Phe Gly His Gly Thr Lys Val Glu Ile  
           115                  120                  125

Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp  
   130                  135                  140

Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn  
  145                  150                  155                  160

Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu  
                   165                  170                  175

Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp  
                   180                  185                  190

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## Claims

1. An antibody against a human CD40 or a functional fragment thereof, having at least one property selected from the following properties (a) to (f) of:

- (a) acting on dendritic cells to produce IL-12 in the presence of LPS and IFN $\gamma$ ;
- (b) having activity to act on dendritic cells causing the cells to mature, which is higher than that of a G28-5 antibody;
- (c) having activity to promote an established B cell line to express CD95, which is higher than that of the G28-5 antibody;
- (d) having activity to suppress the proliferation of an established B cell line, which is higher than that of the G28-5 antibody;
- (e) inducing cell death of an established B cell line; and
- (f) not inhibiting the binding of CD40 ligands to CD40.

2. The antibody or the functional fragment thereof of claim 1, wherein the maturation of dendritic cells is performed at an antibody concentration of 20  $\mu\text{g/ml}$  or less.

3. The antibody or the functional fragment thereof of claim 1, promoting the established B cell line to express CD95 at an antibody concentration of 20  $\mu\text{g/ml}$  or less.

4. The antibody or the functional fragment thereof of claim 1, wherein the established B cell line is Ramos or HS-Sul-ton.

5. The antibody or the functional fragment thereof of claim 1, wherein the production of 100  $\text{pg/ml}$  or more IL-12 is provided when the antibodies with a concentration of 0.1  $\mu\text{g/ml}$  or more are added to dendritic cells with a concentration of  $1 \times 10^6$  cells/ml.

6. The antibody or the functional fragment thereof of claim 1, wherein the production of 1000  $\text{pg/ml}$  or more IL-12 is provided when the antibodies with a concentration of 1  $\mu\text{g/ml}$  or more are added to dendritic cells with a concentration of  $1 \times 10^6$  cells/ml.

7. The antibody or the functional fragment thereof of claim 1, wherein the production of 10000  $\text{pg/ml}$  or more IL-12 is provided when the antibodies with a concentration of 1  $\mu\text{g/ml}$  or more are added to dendritic cells with a concentration of  $1 \times 10^6$  cells/ml.

8. The antibody or the functional fragment thereof of claim 1, promoting within the antibody concentration range between 0.01  $\mu\text{g/ml}$  and 10  $\mu\text{g/ml}$  the established B cell line (Ramos cell) to express CD95 with approximately 2 to 3 times or more greater effectiveness than that expressed by a G28-5 antibody as a control.

9. The antibody or the functional fragment thereof of claim 1, promoting with an antibody concentration of 0.01  $\mu\text{g/ml}$  the established B cell line (Ramos cell) to express CD95 with approximately 2 to 6 times or more greater effectiveness than that expressed by a G28-5 antibody as a control.

10. The antibody or the functional fragment thereof of claim 1, promoting with an antibody concentration of 0.1  $\mu\text{g/ml}$  the established B cell line (Ramos cell) to express CD95 with approximately 2 to 7 times or more greater effectiveness than that expressed by a G28-5 antibody as a control.

11. The antibody or the functional fragment thereof of claim 1, promoting with an antibody concentration of 1  $\mu\text{g/ml}$  the established B cell line (Ramos cell) to express CD95 with approximately 2 to 7 times or more greater effectiveness than that expressed by a G28-5 antibody as a control.

12. The antibody or the functional fragment thereof of claim 1, promoting with an antibody concentration of 10  $\mu\text{g/ml}$  the established B cell line (Ramos cell) to express CD95 with approximately 2 to 6 times or more greater effectiveness than that expressed by a G28-5 antibody as a control.

13. An antibody or a functional fragment thereof, having the amino acid sequences of a heavy chain variable region and a light chain variable region of an antibody that is produced by a hybridoma KM302-1 (Accession No: FERM

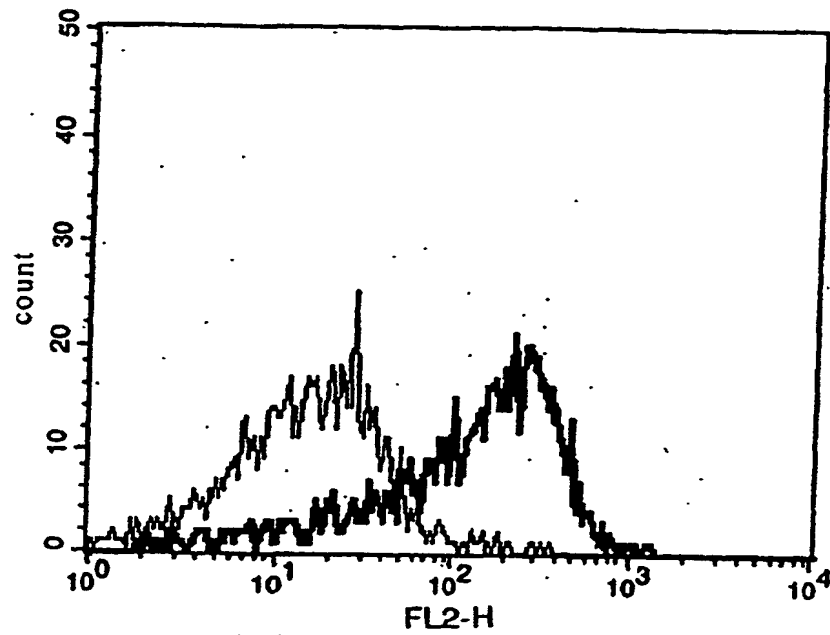
BP-7578), KM341-1-19 (Accession No: FERM BP-7759), 2105 (Accession No: FERM BP-8024) or F1-102 (Accession No: ATCC PTA-3337).

14. An antibody or a functional fragment thereof, having amino acid sequences of the mature portions of a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma F2-103, which are respectively encoded by plasmid DNAs with Accession Nos. ATCC PTA-3302 and ATCC PTA-3303; a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma F5-77, which are respectively encoded by plasmid DNAs with Accession Nos. ATCC PTA-3304 and ATCC PTA-3305; or a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma F5-157, which are respectively encoded by plasmid DNAs with Accession Nos. ATCC PTA-3306 and ATCC PTA-3307.
15. An antibody or a functional fragment thereof, having amino acid sequences of the mature portions of a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma KM341-1-19, which are respectively represented by SEQ ID NOS: 28 and 30; a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma 2105, which are respectively represented by SEQ ID NOS: 32 and 34; a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma 110, which are respectively represented by SEQ ID NOS: 36 and 38; a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma 115, which are respectively represented by SEQ ID NOS: 40 and 42; a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma KM643-4-11, which are respectively represented by SEQ ID NOS: 52 and 54; a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma F2-103, which are respectively represented by SEQ ID NOS: 60 and 62; or a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma F5-77, which are respectively represented by SEQ ID NOS: 64 and 66.
16. An antibody or a functional fragment thereof, having amino acid sequences of the mature portions of a heavy chain variable region and a light chain variable region that are encoded by nucleic acid sequences isolated from a hybridoma KM341-1-19, which are respectively represented by SEQ ID NOS: 27 and 29; a heavy chain variable region and a light chain variable region that are encoded by nucleic acid sequences isolated from a hybridoma 2105, which are respectively represented by SEQ ID NOS: 31 and 33; a heavy chain variable region and a light chain variable region that are encoded by nucleic acid sequences isolated from a hybridoma 110, which are respectively represented by SEQ ID NOS: 35 and 37; a heavy chain variable region and a light chain variable region that are encoded by nucleic acid sequences isolated from a hybridoma 115, which are respectively represented by SEQ ID NOS: 39 and 41; a heavy chain variable region and a light chain variable region that are encoded by nucleic acid sequences isolated from a hybridoma KM643-4-11, which are respectively represented by SEQ ID NOS: 51 and 53; a heavy chain variable region and a light chain variable region that are encoded by nucleic acid sequences isolated from a hybridoma F2-103, which are respectively represented by SEQ ID NOS: 59 and 61; or a heavy chain variable region and a light chain variable region that are encoded by nucleic acid sequences isolated from a hybridoma F5-77, which are respectively represented by SEQ ID NOS: 63 and 65.
17. An antibody against a human CD40, or a functional fragment thereof, having at least one property selected from the following properties (g) to (j) of:
  - (g) neutralizing the action of ligands for CD40;
  - (h) neutralizing or alleviating one or more effects that ligands, which are for CD40 on an established B cell line, have on CD40-expressing cells, and having agonistic action on CD40 on the established B cell line weaker than that of 5D12 due to cross-linking by anti-immunoglobulin antibodies;
  - (i) alleviating or neutralizing the action of CD40 ligands on the established B cell line to increase CD95 expression; and
  - (j) having antagonistic action on CD40 expressed on dendritic cells.
18. The antibody or the functional fragment of claim 17, which can suppress the expression of CD95 in Ramos cells to a level approximately 10% or less than that of a control, when antibodies with a concentration of 0.1 µg/ml are added to the Ramos cells with a concentration of  $1 \times 10^6$  cells/ml supplemented with a saturated amount of CD40L-expressing cells.
19. The antibody or the functional fragment of claim 17, which can suppress the expression of CD95 in Ramos cells to the same level as that of a negative control, when the antibodies with a concentration of 1 µg/ml are added to the Ramos cells with a concentration of  $1 \times 10^6$  cells/ml supplemented with a saturated amount of CD40L-express-

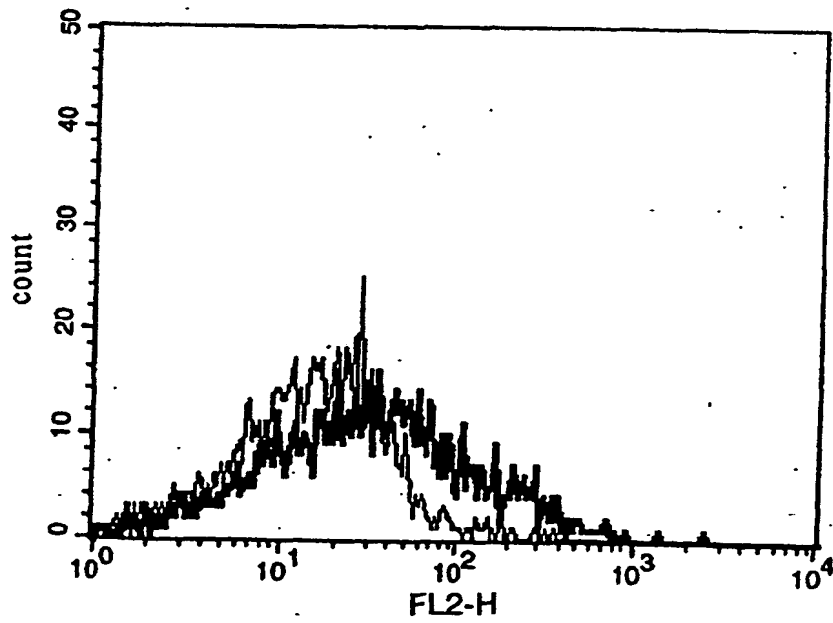
ing cells.

20. The antibody or the functional fragment of claim 17, which can suppress the expression of CD95 in the Ramos cells to the same level as that of the negative control, when the antibodies with a concentration of 10 µg/ml are added to the Ramos cells with a concentration of  $1 \times 10^6$  cells/ml supplemented with a saturated amount of CD40L-expressing cells.
21. The antibody or the functional fragment of claim 17, wherein the proliferation of tonsillar B cells is suppressed in vitro by approximately 80 to 95% or more, when the antibodies with a concentration between 0.001 µg/ml and 10 µg/ml are added to  $1 \times 10^5$  tonsillar B cells supplemented with soluble CD40L (1µg/ml).
22. The antibody or the functional fragment of claim 17, wherein the proliferation of tonsillar B cells is suppressed in vitro by approximately 95% or more, when the antibodies with a concentration between 0.01 µg/ml and 10 µg/ml are added to  $1 \times 10^5$  tonsillar B cells supplemented with soluble CD40L (1µg/ml).
23. The antibody or the functional fragment of claim 17, wherein the proliferation of tonsillar B cells is suppressed in vitro by approximately 80% or more, when the antibodies with a concentration of 0.001 µg/ml are added to  $1 \times 10^5$  tonsillar B cells supplemented with soluble CD40L (1µg/ml).
24. An antibody or a functional fragment thereof, having amino acid sequences of a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma KM281-1-10 (Accession No: FERM BP-7579), 4D11 (Accession No: FERM BP-7758) or F4-465 (Accession No: ATCC PTA-3338).
25. An antibody or a functional fragment thereof, having amino acid sequences of the mature portions of a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma KM281-1-10, which are respectively represented by SEQ ID NOS: 44 and 46; a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma 4D11, which are respectively represented by SEQ ID NOS: 48 and 50; or a heavy chain variable region and a light chain variable region of the antibody produced by a hybridoma F4-465, which are respectively represented by SEQ ID NOS: 56 and 58.
26. An antibody or a functional fragment thereof, having amino acid sequences of the mature portions of a heavy chain variable region and a light chain variable region that are encoded by nucleic acid sequences isolated from a hybridoma KM281-1-10, which are respectively represented by SEQ ID NOS: 43 and 45; a heavy chain variable region and a light chain variable region of an antibody produced by a hybridoma 4D11, which are respectively represented by SEQ ID NOS: 47 and 49; or a heavy chain variable region and a light chain variable region that are encoded by nucleic acid sequences isolated from a hybridoma F4-465, which are respectively represented by SEQ ID NOS: 55 and 57.
27. The antibody or the functional fragment thereof of any one of claims 1 to 26, which is a human antibody.
28. A pharmaceutical composition, containing as an active ingredient the antibody or the functional fragment thereof of any one of claims 1 to 26.
29. An immunopotentiating agent, anti-tumor agent or anti-autoimmune disease agent, containing as an active ingredient the antibody or the functional fragment thereof of any one of claims 1 to 16.
30. An immunosuppressive agent, anti-autoimmune disease agent, therapeutic agent against allergies or therapeutic agent against blood coagulation factor VIII -inhibiting syndrome, containing as an active ingredient the antibody or the functional fragment thereof of any one of claims 17 to 26.

Fig. 1

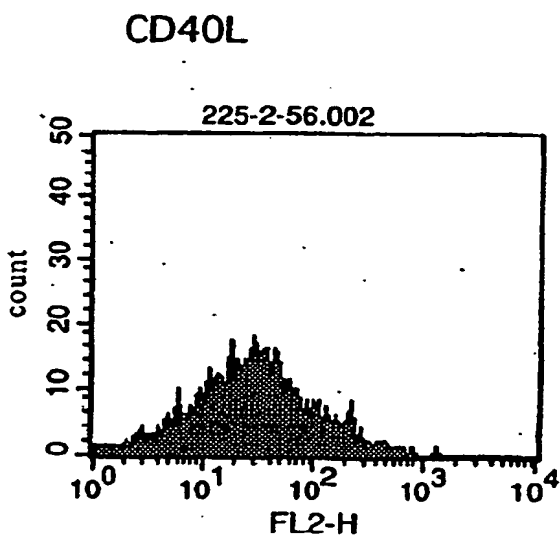
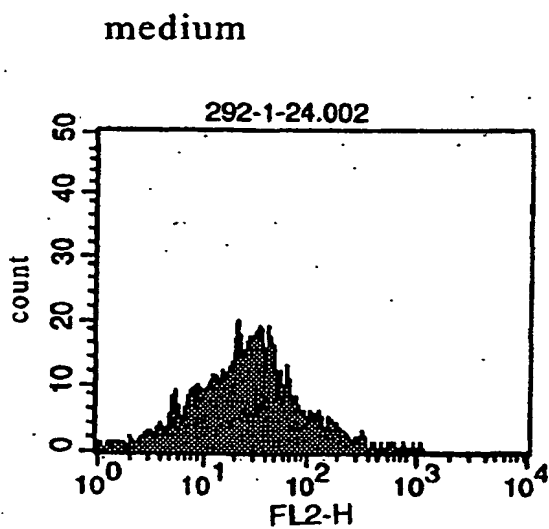
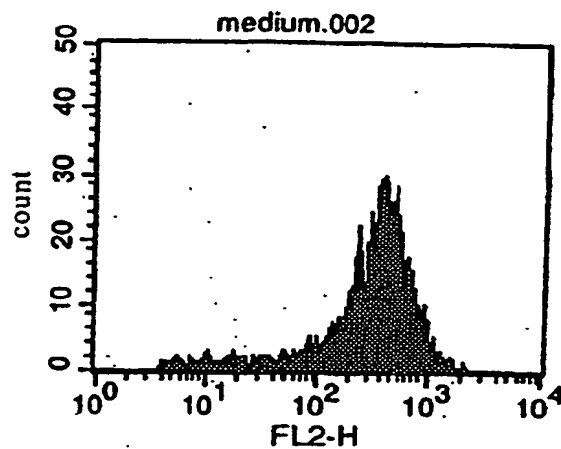
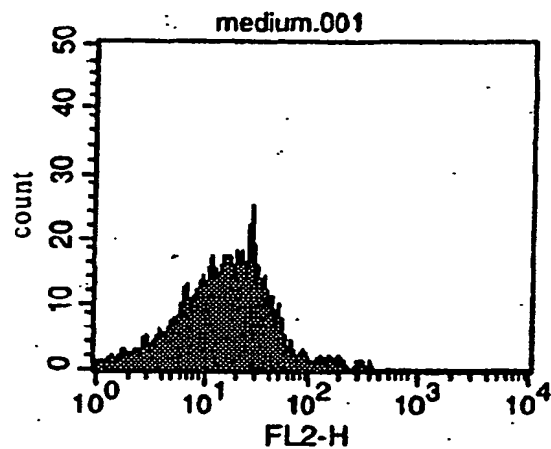


KM302-1 antibody



G28-5 antibody

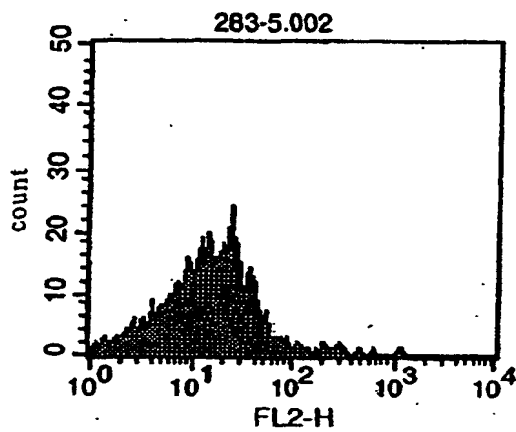
Fig. 2A



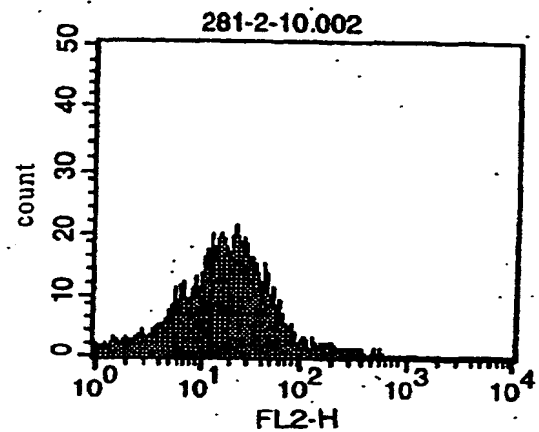
KM292-1-24 antibody

KM225-2-56 antibody

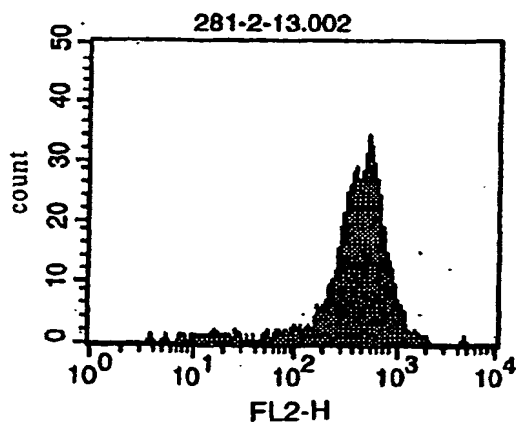
Fig. 2B



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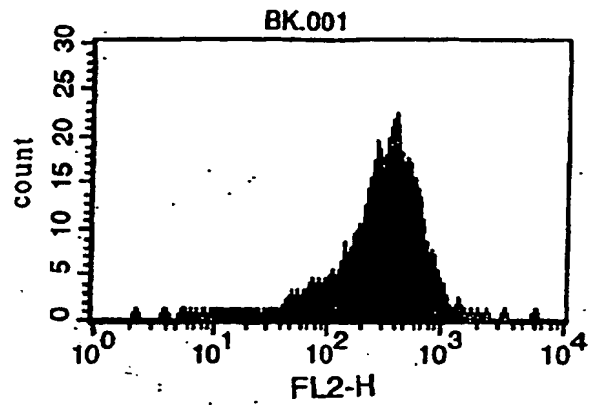


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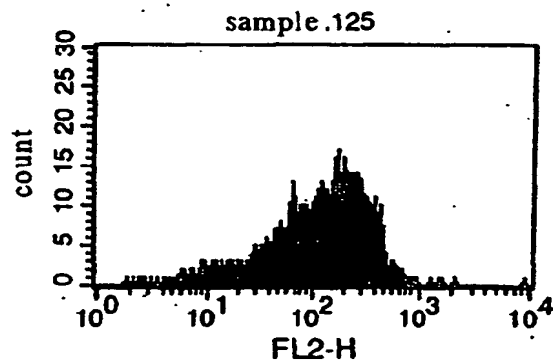


KM281-2-13 antibody

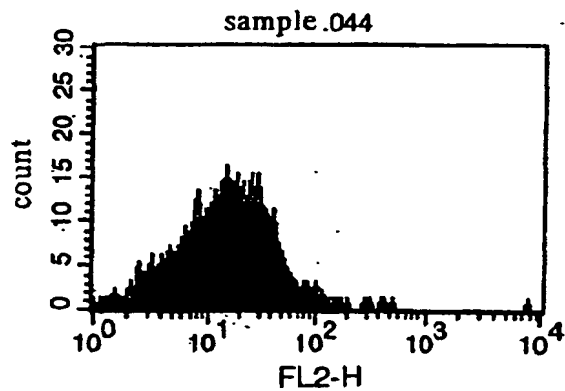
Fig. 3



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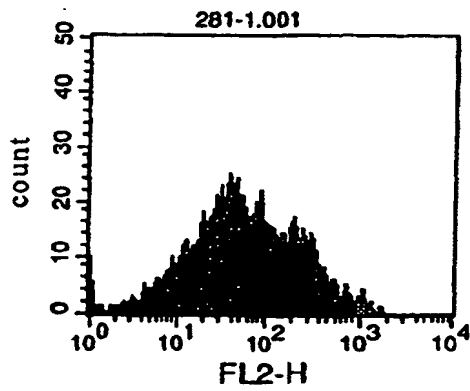


CD40L+5D12

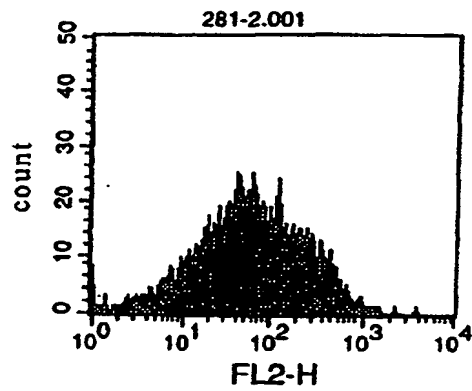


CD40L+KM281-1-10 antibody

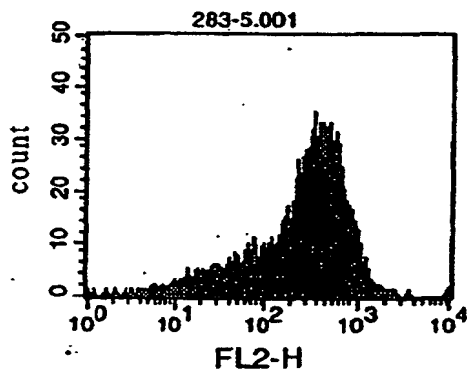
Fig. 4



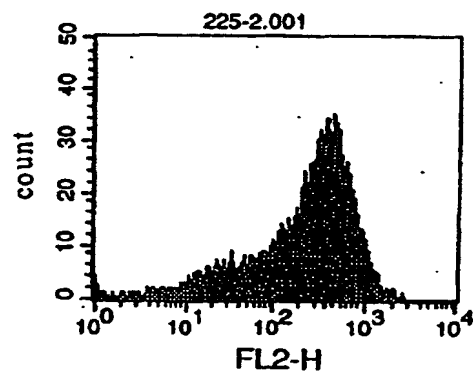
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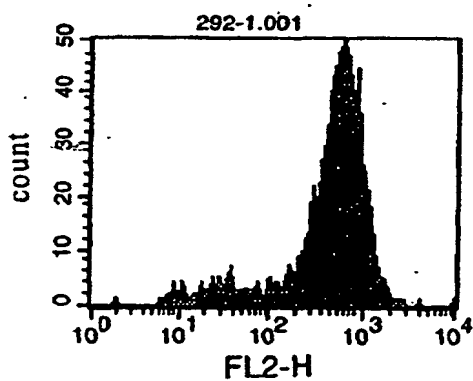
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KM283-5 antibody



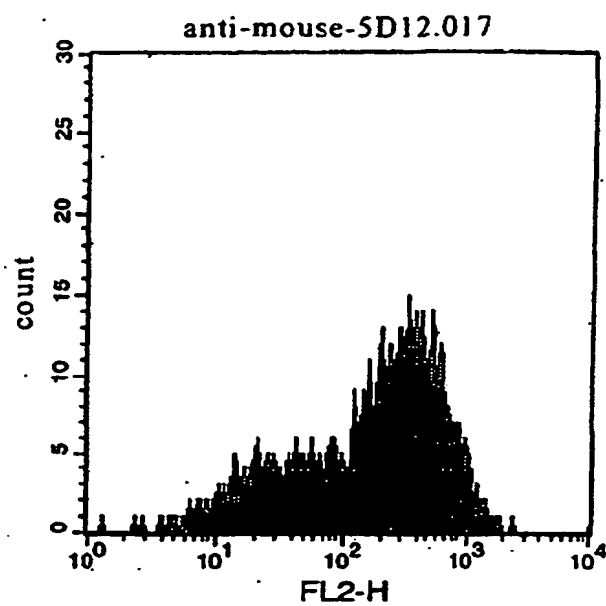
KM225-2-56 antibody



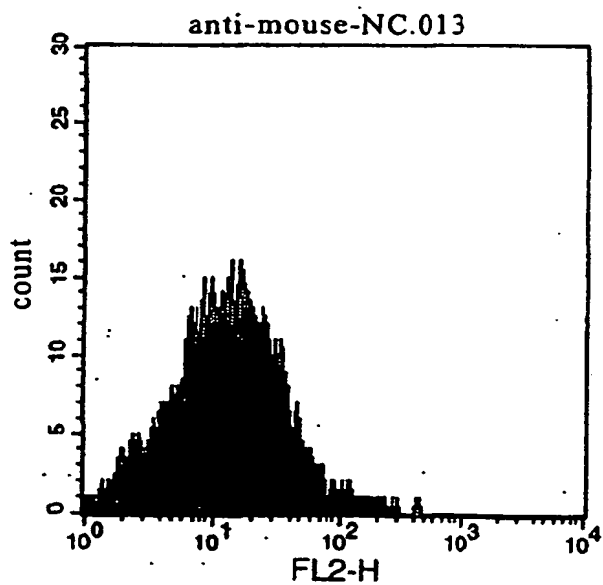
KM292-1-24 antibody



Fig. 5



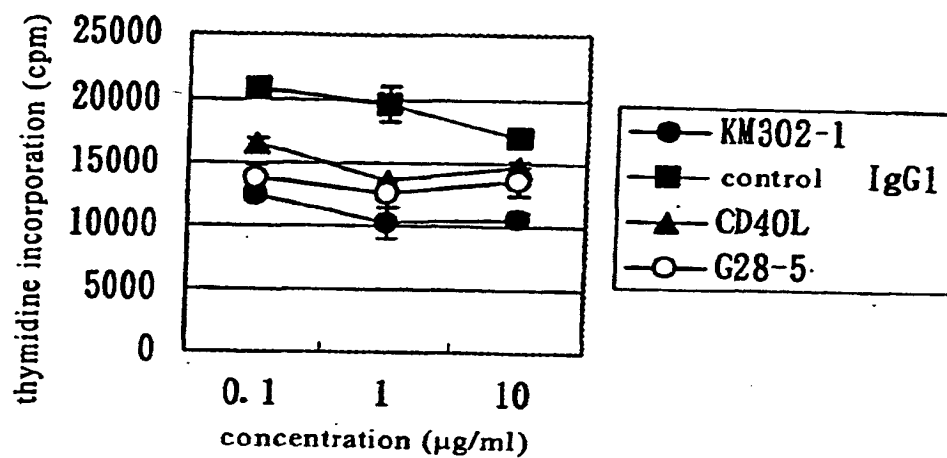
5D12 antibody+anti-mouse IgG antibody



anti-mouse IgG antibody only

Fig. 6

HS-Sulton



Ramos

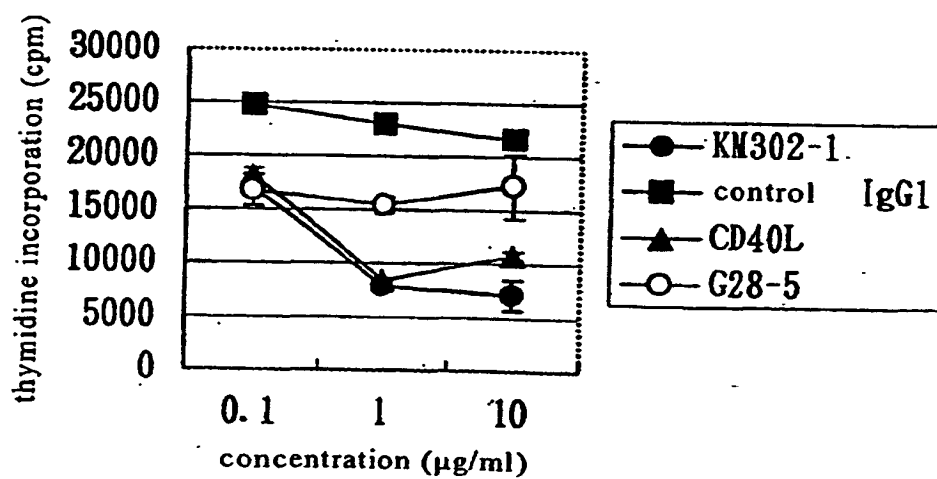
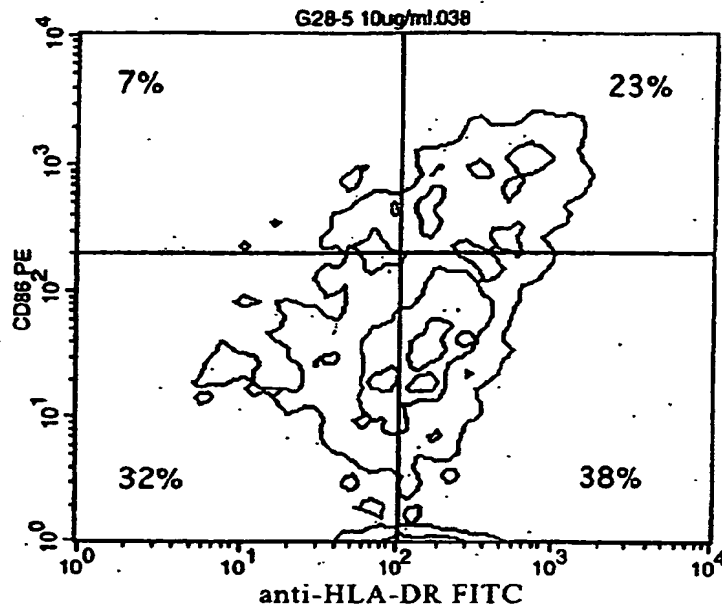
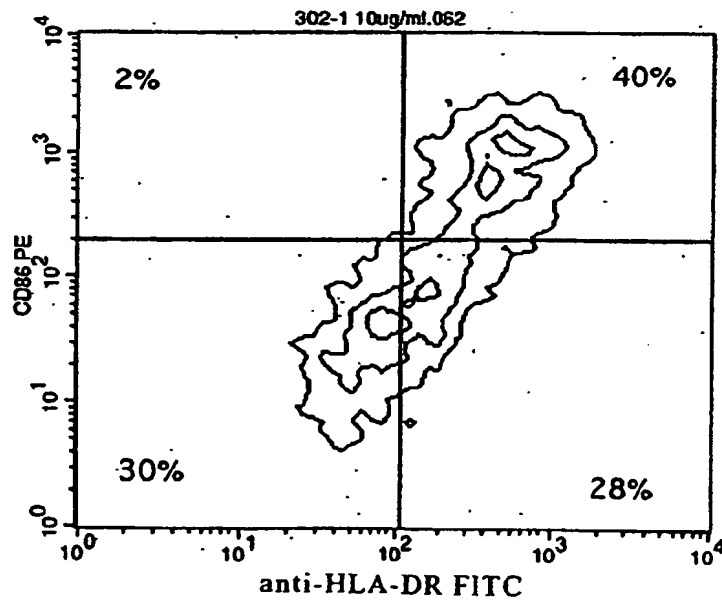


Fig. 7



G28-5 antibody



KM302-1 antibody

Fig. 8

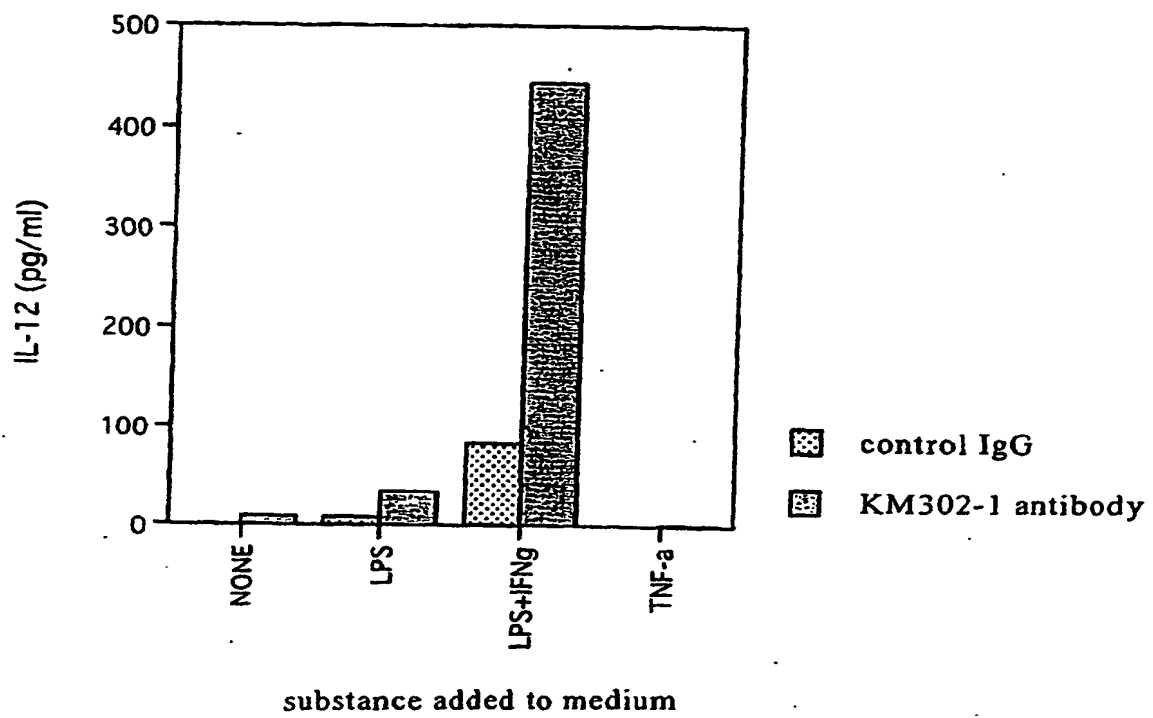


Fig.9

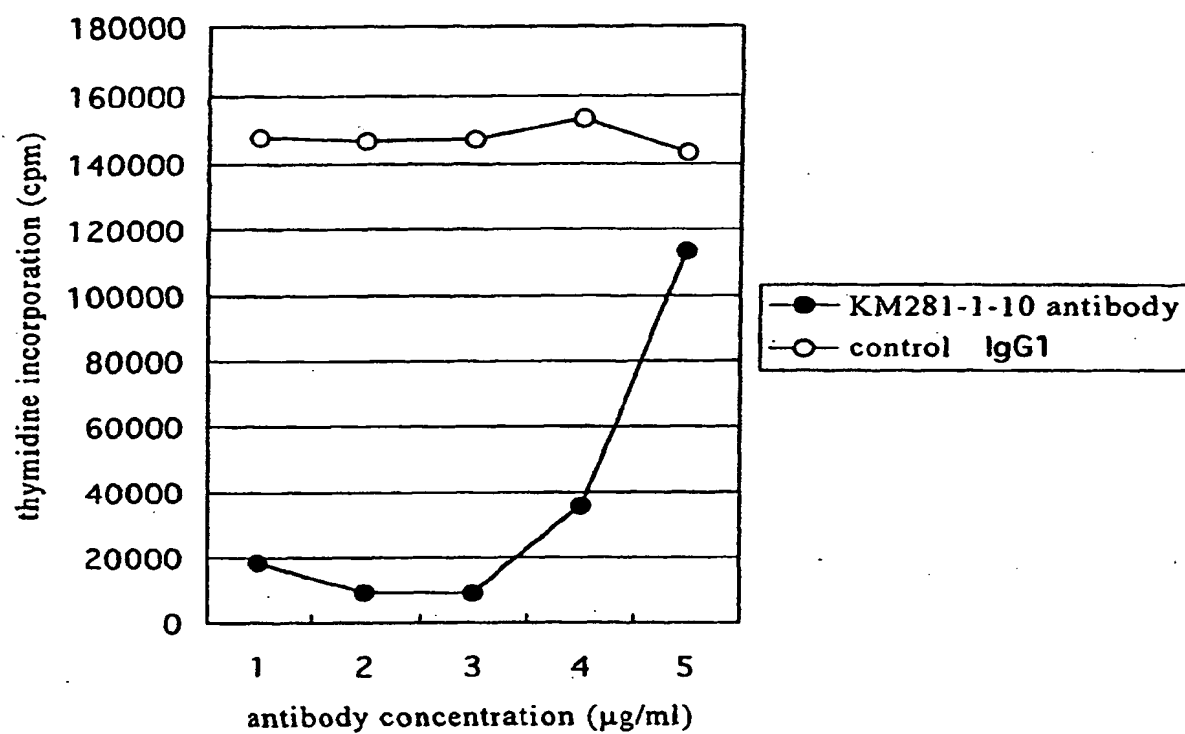


Fig. 10

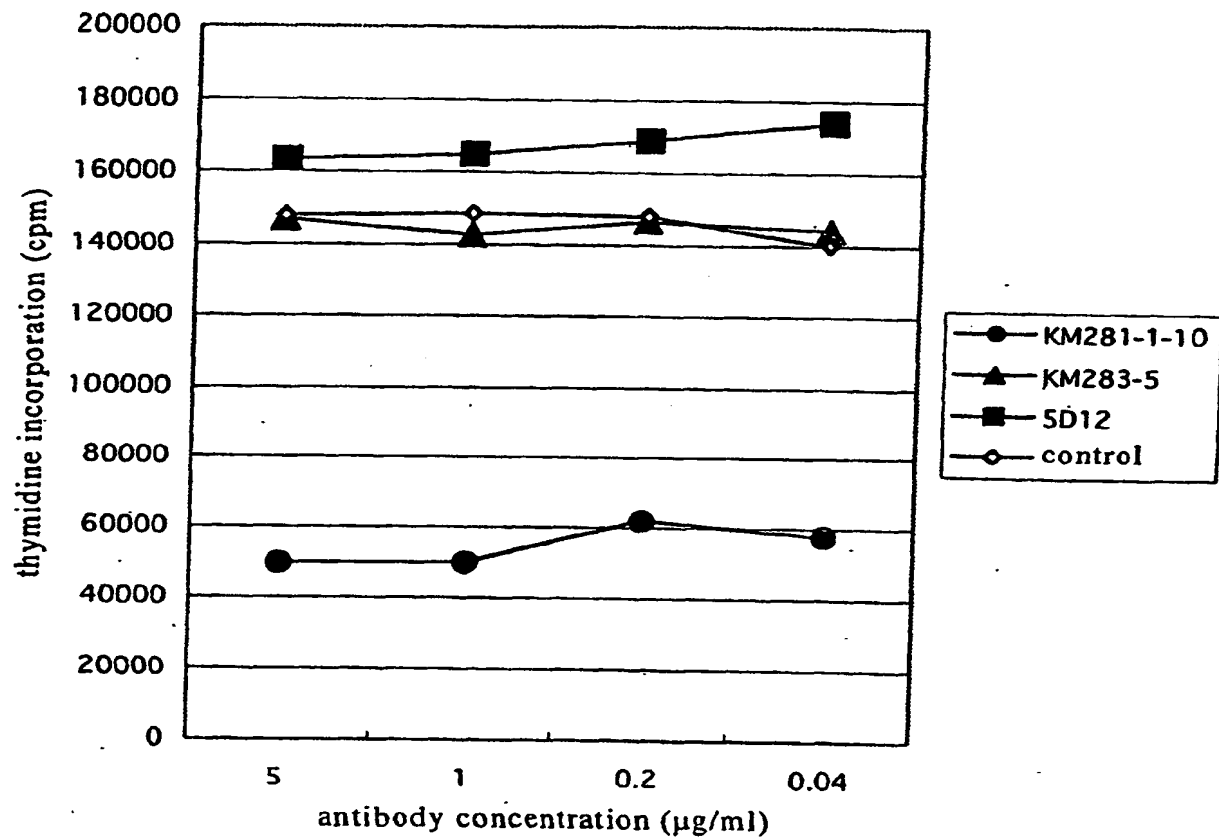


Fig. 11

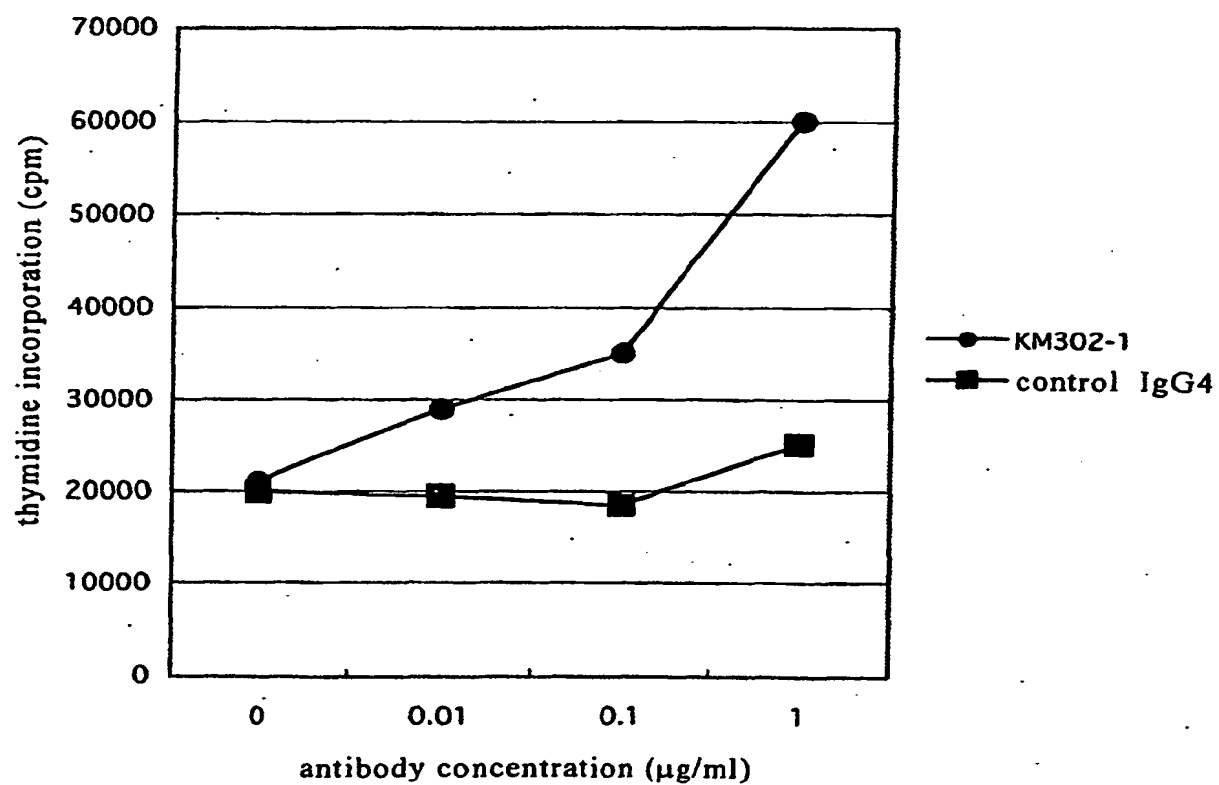


Fig. 12

Action of anti-CD40 agonistic antibody to increase CD95 expression in Ramos cell

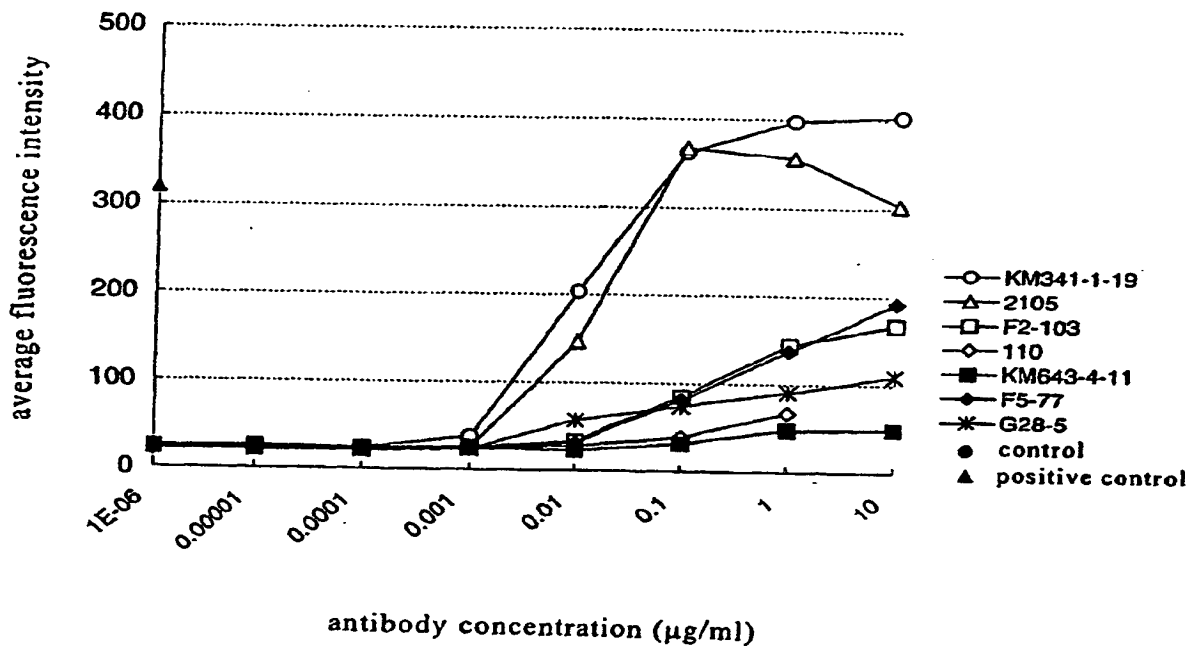




Fig. 13

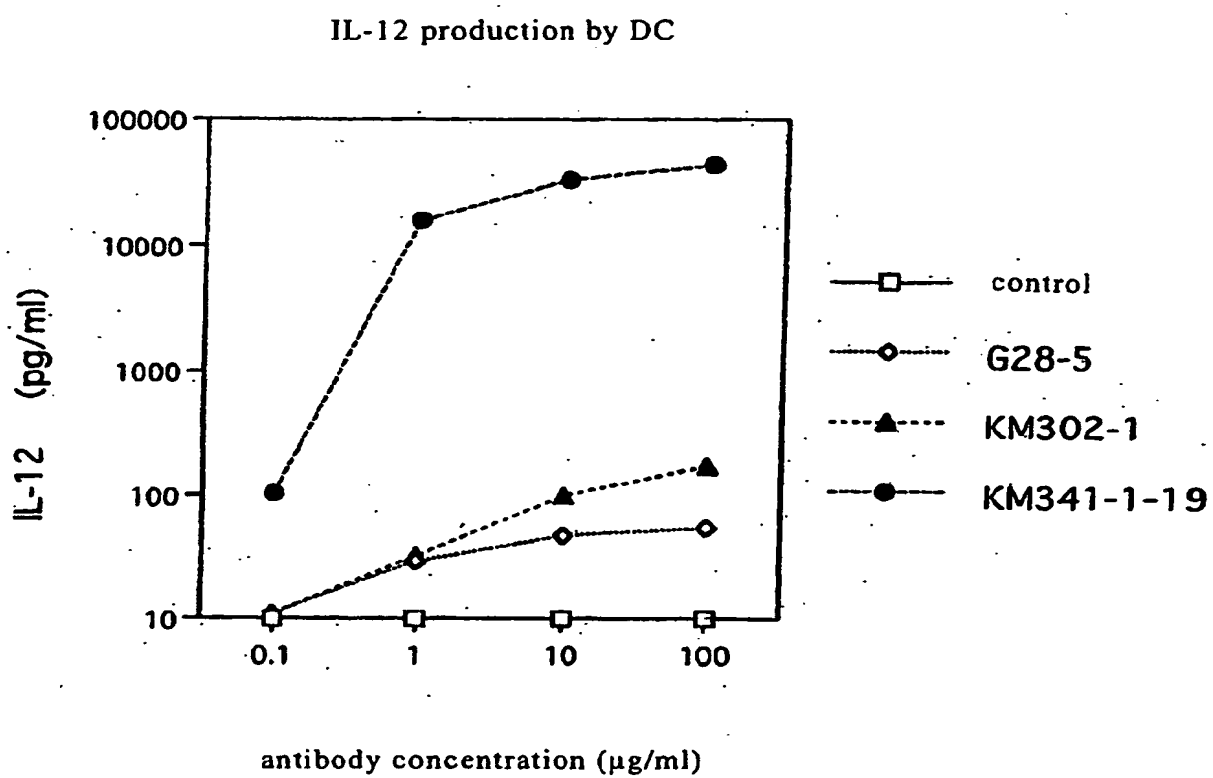


Fig. 14

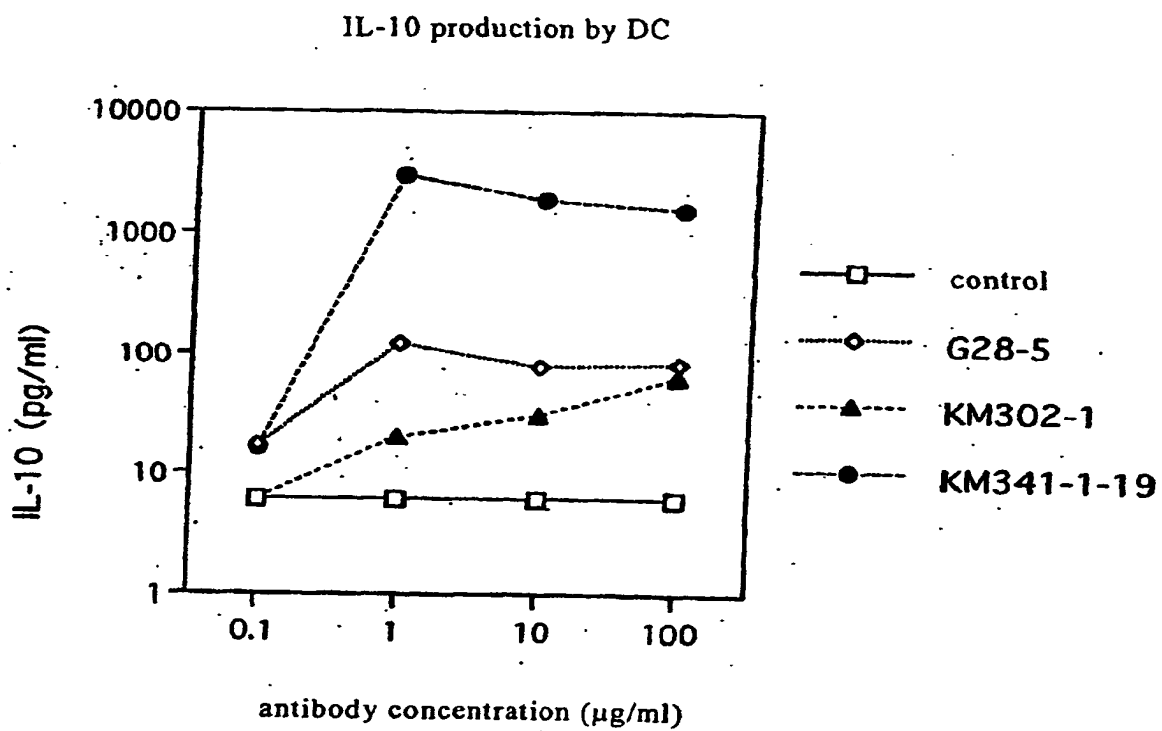


Fig. 15

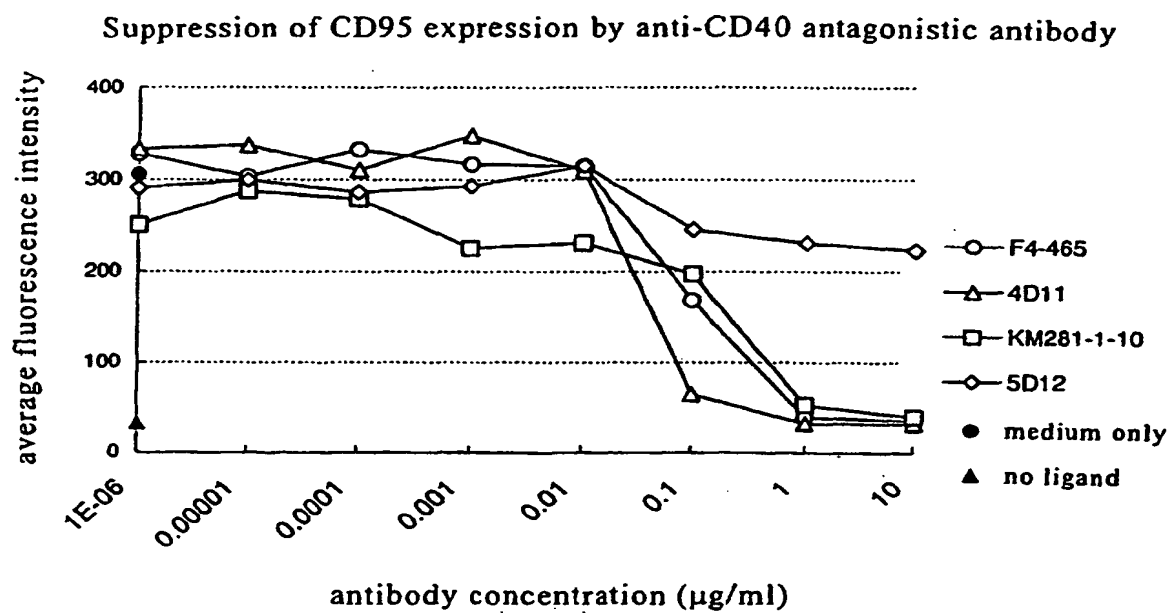


Fig. 16

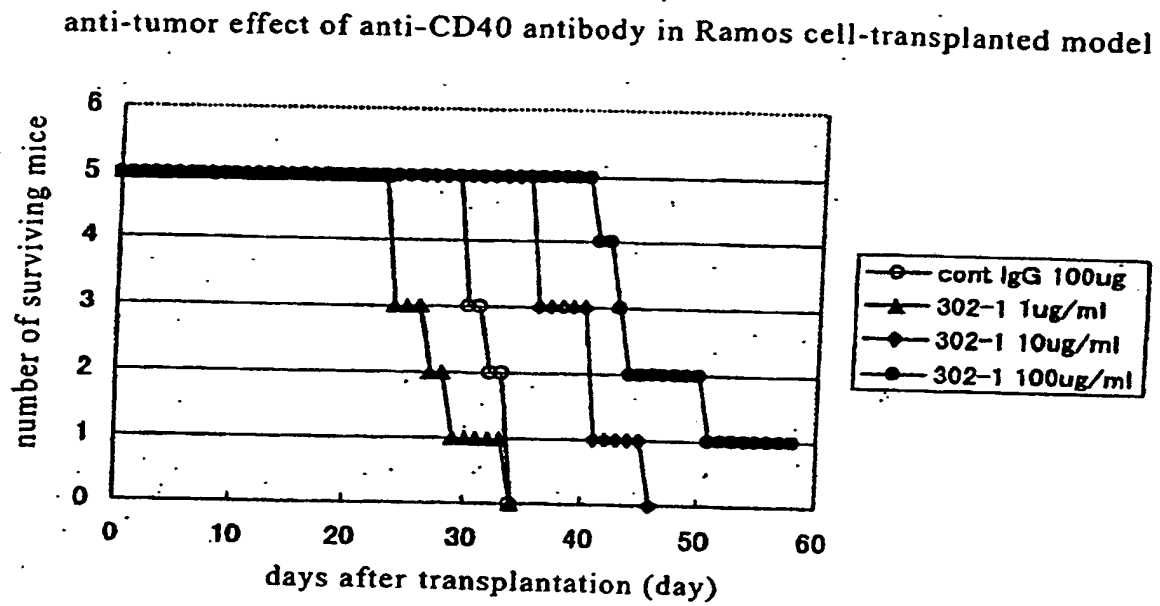


Fig. 17A

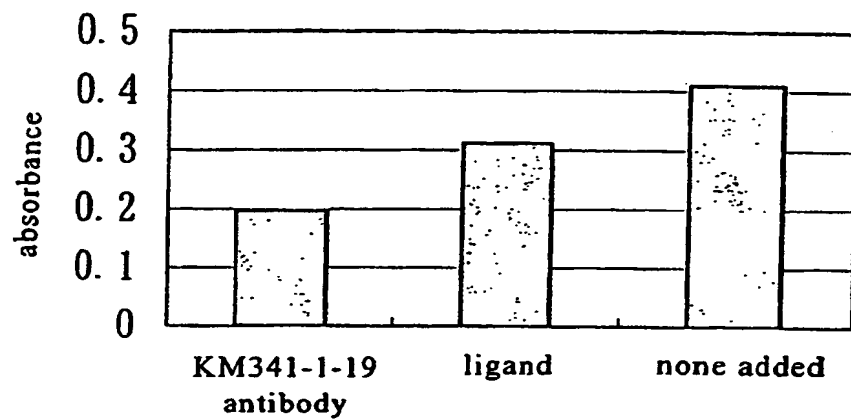


Fig. 17B

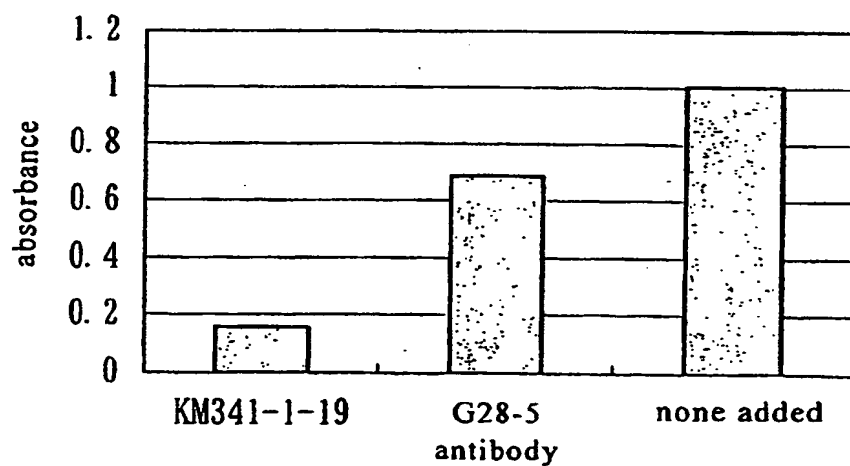


Fig. 18

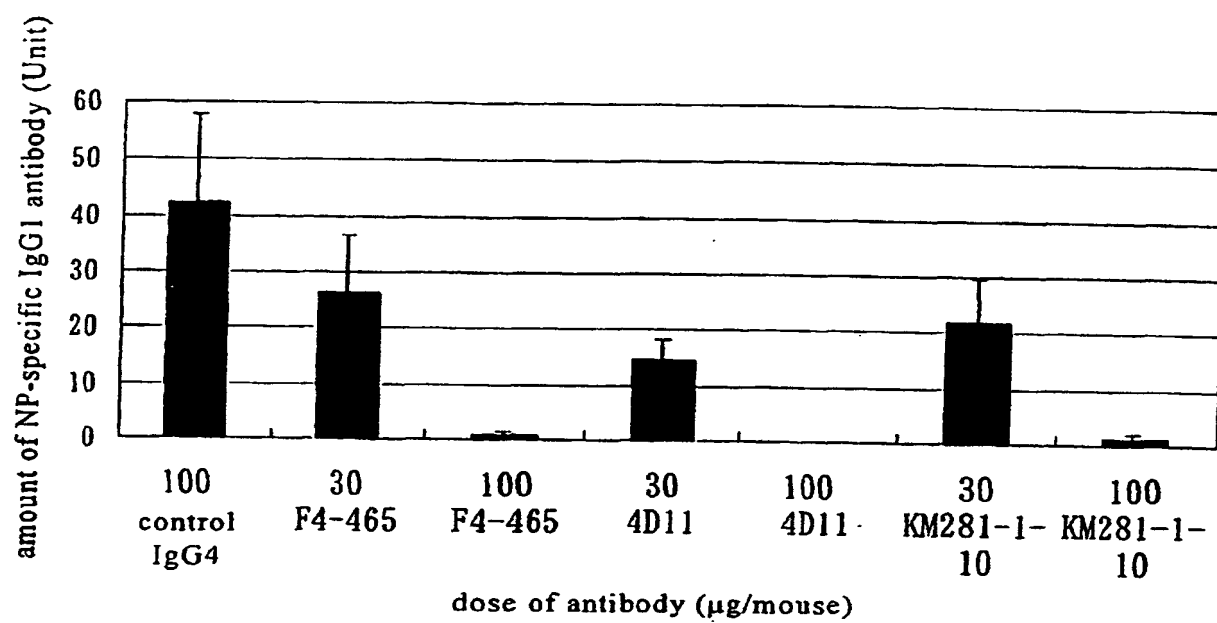


Fig. 19

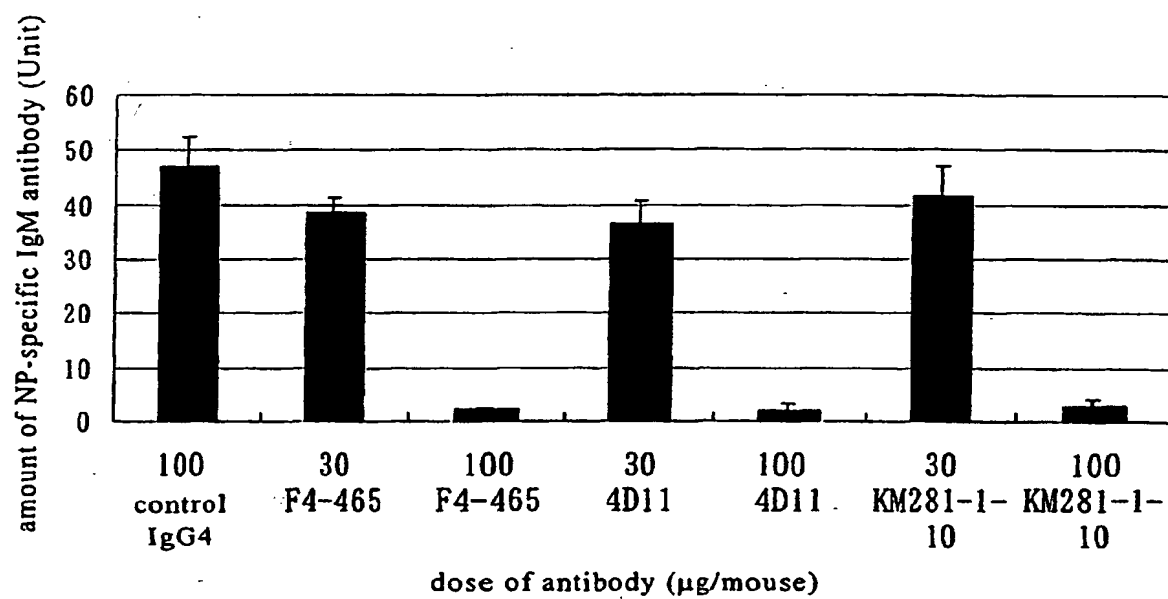
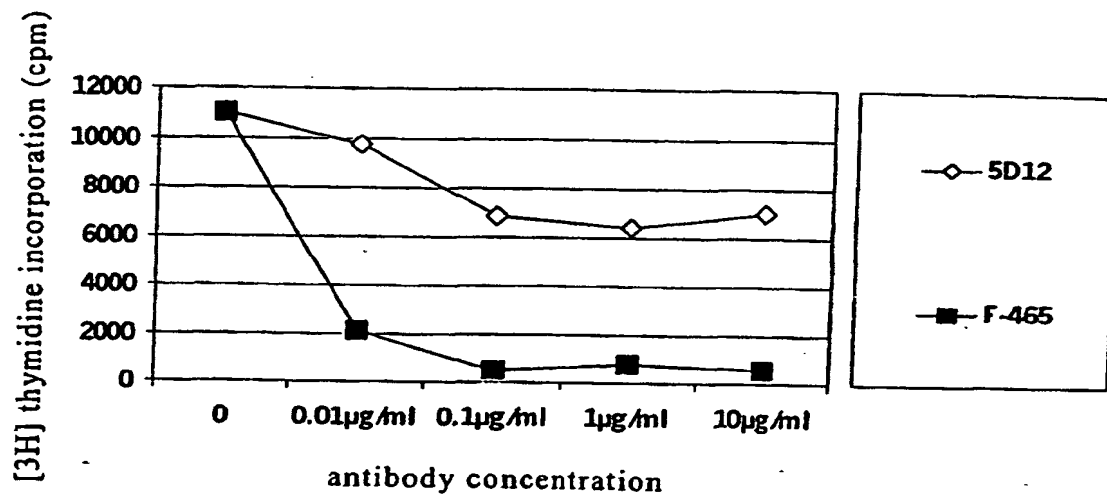


Fig. 20





## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP02/04292

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
Int.Cl <sup>7</sup> C07K16/28, C12N15/13, C12N5/10, C12P21/08, A61K39/395, A61P35/00, A61P37/04, A61P37/06, A61P37/08, A61P7/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols)		
Int.Cl <sup>7</sup> C07K16/28, C12N15/13, C12N5/10, C12P21/08, A61K39/395, A61P35/00, A61P37/04, A61P37/06, A61P37/08, A61P7/00		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
SwissProt/PIR/Geneseq, WPI(DIALOG), BIOSIS(DIALOG), JICST FILE(JOIS)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Ledbetter, J. A. et al., Agonistic activity of a CD40-specific single-chain Fv constructed from the variable regions of mAb G28-5. Crit. Rev. Immunol., 1997, Vol.17, Nos.5 to 6, pages 427 to 435	1-16, 28, 29 27
X Y	Zhou, Z. H. et al., An agonist anti-human CD40 monoclonal antibody that induces dendritic cell formation and maturation and inhibits proliferation of a myeloma cell line. Hybridoma, Vol.18, No.6, pages 471 to 478(1999)	1-16, 28, 29 27
X Y	de Boer, M. et al., Generation of monoclonal antibodies to human lymphocyte cell surface antigens using insect cells expressing recombinant proteins. J. Immunol. Methods, Vol.152, No.1, pages 15 to 23(1992)	17-26, 28, 30 27
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>		
Date of the actual completion of the international search 08 July, 2002 (08.07.02)		Date of mailing of the international search report 23 July, 2002 (23.07.02)
Name and mailing address of the ISA/ Japanese Patent Office		Authorized officer
Facsimile No.		Telephone No.

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP02/04292

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	Boon, L. et al., Preclinical assessment of anti-CD40 Mab 5D12 in cynomolgus monkeys. Toxicology, Vol.174, pages 53 to 65 (2002)	17-26, 28, 30
Y	Tomizuka, K. et al., Double trans-chromosomic mice: Maintenance of two individual human chromosome fragments containing Ig heavy and Ioci and expression of fully human antibodies. Proc. Natl. Acad. Sci. USA, Vol.97, No.2, pages 722 to 727 (2000)	27
Y	WO 98/37757 A1 (Kirin Beer), 03 September, 1998 (03.09.98), & EP 972445 A1	27

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP02/04292

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A61P35/00, A61P37/04, A61P37/06, A61P37/08, A61P7/00

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☒ Further documents are listed in the continuation of Box C.

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\* Special categories of cited documents:

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

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## INTERNATIONAL SEARCH REPORT

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Y	WO 98/37757 A1 (Kirin Beer), 03 September, 1998 (03.09.98), & EP 972445 A1	27

## A. 発明の属する分野の分類 (国際特許分類 (IPC))

Int. Cl<sup>7</sup> C07K16/28, C12N15/13, C12N5/10, C12P21/08, A61K39/395,  
A61P35/00, A61P37/04, A61P37/06, A61P37/08, A61P7/00

## B. 調査を行った分野

## 調査を行った最小限資料 (国際特許分類 (IPC))

Int. Cl<sup>7</sup> C07K16/28, C12N15/13, C12N5/10, C12P21/08, A61K39/395,  
A61P35/00, A61P37/04, A61P37/06, A61P37/08, A61P7/00

最小限資料以外の資料で調査を行った分野に含まれるもの

国際調査で使用した電子データベース (データベースの名称、調査に使用した用語)

SwissProt/PIR/Geneseq,  
WPI(DIALOG)、BIOSIS(DIALOG)、JICSTファイル(JOIS)

## C. 関連すると認められる文献

引用文献の カテゴリー*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する 請求の範囲の番号
X	Ledbetter, J. A. et al., Agonistic activity of a CD40-specific single-chain Fv	1-16, 28, 29
Y	constructed from the variable regions of mAb G28-5. Crit. Rev. Immunol., 1997, Vol. 17, No. 5-6, pp. 427-435	27
X	Zhou, Z. H. et al., An agonist anti-human CD40 monoclonal antibody that induces	1-16, 28, 29
Y	dendritic cell formation and maturation and inhibits proliferation of a myeloma cell line. Hybridoma, Vol. 18, No. 6, pp. 471-478 (1999)	27

☒ C欄の続きにも文献が列挙されている。

☐ パテントファミリーに関する別紙を参照。

## \* 引用文献のカテゴリー

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「P」 国際出願日前で、かつ優先権の主張の基礎となる出願

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「&」 同一パテントファミリー文献

国際調査を完了した日

08.07.02

国際調査報告の発送日

23.07.02

国際調査機関の名称及びあて先

日本国特許庁 (ISA/J P)

郵便番号 100-8915

東京都千代田区霞が関三丁目4番3号

特許庁審査官 (権限のある職員)

田村 明 照

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電話番号 03-3581-1101 内線 3448

C (続き) . 関連すると認められる文献		
引用文献の カテゴリー*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する 請求の範囲の番号
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Y	Tomizuka, K. et al., Double trans-chromosomic mice: Maintenance of two individual human chromosome fragments containing Ig heavy and loci and expression of fully human antibodies. Proc. Natl. Acad. Sci. USA, Vol. 97, No. 2, pp. 722-727 (2000)	27
Y	WO 98/37757 A1 (Kirin Beer) 1998.09.03 & EP 972445 A1	27

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